UNIVERSITY OF ALBERTA

ETHANOL AND SUPERCRITICAL FLUID EXTRACTION OF *ECHINACEA ANGUSTIFOLIA* ROOTS

BY

LI SUN

A THESIS SUMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFULLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

FOOD SCIENCE AND TECHNOLOGY

DEPARTEMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE

EDMONTON, ALBERTA

FALL, 2001

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DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 2001

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ABSTRACT

Echinacoside and cynarine in dried *Echinacea angustifolia* roots were extracted using pure ethanol (100%) at 25, 50 and 75°C for 30, 60 and 90 min with solvent to feed ratios of 20:1 and 10:1. Echinacoside and cynarine yields were significantly affected ($p \leq 0.05$) by all three variables and increased with increasing temperature and time. Higher solvent to feed ratio generally resulted in higher yields. The highest echinacoside and cynarine yields obtained were 0.82% and 1.10%, respectively.

Alkylamides in fresh and dried *E. angustifolia* roots were extracted using supercritical carbon dioxide at 45, 60°C and 34, 55 MPa. Alkylamide yield of fresh roots increased with increasing temperature but decreased with increasing pressure while those from ground air-dried roots increased with higher temperature and pressure. The latter yielded the highest alkylamides as compared with those from fresh or unground dried roots. The use of freeze-dried roots did not give better extraction results as compared to air-dried roots for echinacoside, cynarine and alkylamides.

ACKNOWLEDGEMENT

Praise the Lord who brings me to Canada. I would like to thank my supervisors, Dr. Ooraikul and Dr. Temelli, for accepting me as their student. I am very grateful for their guidance and patience during my study and thesis work. They have encouraged me to pursue my graduate study and given me a lot of advise on various issues. Under their supervision, I am growing not only in food science knowledge and skills, but also in personalities.

My many thanks also extent to Dr. Hoyano, Dr. Habil, Dr. Rezaei and Dr. Nakano, who had helped and advised me while I was conducting the lab work at Longman, AVEC and on campus, respectively.

I also own great debts to my research colleagues, Stephanie Shum, S.Y. Li, Gorden, Neil, Tipawan and Sing, who offered great help at various stages of the experiment and thesis writing.

C.P. Xu, my husband, had supported and cared for me during my entire study. My parents and brother in China also encouraged me whenever they had the chance. I could not finish graduate study successfully without family harmony. I want to say thank you all, my family members, and I would like to share this honour with you all.

May God continue to bless University of Alberta and us!

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. GENERAL INTRODUCTION AND OBJECTIVES

1.1.1. Introduction

The focus of the new approaches in nutrition has been shifting from treatment of disease to prevention of disease over the last decade. The focus has moved from the former emphasis on treatment of malnutrition to the present emphasis on the potential of foods to promote health, for improving both mental and physical well being and reducing the risk of diseases. The new concepts of nutrition stimulate and support research on the physiological effects of food components and their health benefits (Diplock *et al.*, 2000). In USA, 'reduction of disease risk' claims have been allowed since 1993. The most recent approved claim was on the soy protein for its effect on reducing the risk of heart disease (FDA, 1999).

Consumer awareness of nutritional science is growing. There is increased expectation of obtaining health benefits from foods. This has created a challenge as well as an opportunity for the food scientists and the industry.

An old Chinese saying 'herbal medicine and food cannot be separated from one family' demonstrates the thinking of the ancient Chinese people on the rules of food and herbal medicine to maintain good health. Indeed, plants provide human beings with a wide variety of nutritive, health-promoting and therapeutic ingredients. Culinary herbs and spices are rich sources of phytochemicals, many of which have distinctly strong,

aromatic and bitter taste and flavor (Walker, 2000). Rich sources of phytochemicals are also found among the tonic herbal medicines and they are also appearing as functional ingredients in traditional foods (Brevoort, 1998). The application of phytotherapy (herbal medicine) using the tonic herbs would result in benefits to all cells of the body (Walker, 2000). Phytotherapy accounted for approximately 7% of all prescription medications covered by public health insurance in Germany in 1995, with total sales of about 2 billion DM (Schulz *et al.*, 1998).

It is not only in Germany and European countries; herb sales are also strong in the United States. Among the 46 herbs listed in the 4th annual natural herbal products sales survey conducted by the *Whole Foods* magazine in 1998, Echinacea products from the American native herbal plant, received the number one spot for sales value in herbal supplements for the 4th consecutive year. The Echinacea products represented 9.79% of total herbal supplement sales. At the same time, foods and beverages containing herbs captured 6.12% of total store sales (Richman and Witkowski, 1998).

Echinacea is native to North America. It has been well recognized for its effectiveness in treating inflammations and injuries of skin as well as being a non-specific immune stimulant (Foster, 1991; Schulz *et al.*, 1998; Bauer, 1998a, b).

Currently, three out of the nine Echinacea species are cultivated and used in various pharmaceutical products (Cullen, 1990). They are *Echinacea angustifolia*, *E. purpurea* and *E. pallida*. Among them, *E. angustifolia* was the most widely used species as medicine by the Native Indians and was the first species utilized commercially by H.C.F. Meyer, a German physician in early 1900s. John Uri Lloyd, a physician from a

group of 'Eclectics' doctors, started the first commercial pharmaceutical preparations of Echinacea in the United States (Foster, 1991; Bauer, 1998a; Schulz *et al.*, 1998).

It is believed that there are several active components of Echinacea, belonging to the classes of polysaccharides, caffeic acid derivatives (cichoric acid and echinacoside) and alkylamides, acting as a whole and contributing to the immunostimulatory activity of Echinacea (Bauer, 1998a).

Traditional procedures used for the isolation of phytochemicals from plant materials for therapeutic purposes include aqueous and aqueous-alcohol extraction, even though the specific components that determine the pharmacological activity are unknown (Bombardelli, 1991; Schulz *et al.*, 1998). In the case of Echinacea, many studies have been conducted on the isolation of different compounds with an attempt to better understand its antiviral, immune stimulating and antibacterial properties (Stroll *et al.*, 1950; Jacobson, 1954, 1967; Bauer *et al.*, 1988b, 1989b; Bauer and Wagner, 1991; Bauer, 1998a, b). The analysis of active components and methods to differentiate species have been well developed by a number of research groups (Bauer *et al.*, 1988a,b, 1989b; Bauer and Remiger, 1989; Perry *et al.*, 1997; He *et al.*, 1998; Lienert *et al.*, 1998; Pietta *et al.*, 1998).

Echinacea is a herb in high demand and efficient extraction of its components is essential. Solvent extraction is widely used in the food and pharmaceutical industries. Ethanol or aqueous ethanol is the solvent of choice for phytomedicinal production of extracts. Supercritical fluid extraction, using gases under supercritical conditions, is a promising technology in food and pharmaceutical industries. It has advantages over solvent extraction by producing cleaner extracts, having minimal operation steps and

using environmentally friendly solvents. Even though there are hundreds of Echinacea products sold in the form of extracts, capsules, tablets, teas or tinctures, the effect of extraction conditions on the recovery of active components such as alkylamides and two caffeic acid derivatives, echinacoside and cynarine, has not been well defined. This study was therefore designed to fill this gap in knowledge.

1.1.2. Objectives

The primary objectives of the present thesis work are:

1. To determine the effect of extraction conditions on moderately polar components, echinacoside and cynarine, using ethanol as solvent, from *E. angustifolia* roots, and
2. To determine the effect of extraction conditions on non-polar components, alkylamides, using supercritical CO_2 as solvent, from *E. angustifolia* roots.

1.2. ECHINACEA

Echinacea is a genus of the *Compositae* (or *Asteraceae)* botanical family (Bauer and Wagner, 1991; Hobbs, 1994; Leung and Foster, 1996). There are nine species (Hobbs, 1989; Ellyett and Morey, 1996) in this family but only three have demonstrated healing properties, i.e. *Echinacea angustifolia*, *E. purpurea* and *E. pallida*. The first two species are favored for commercial use (Cullen, 1990; Davies, 1999).

Echinacea is hardy and well adaptable to frost and drought (Hobbs, 1989). It grows wild in the dry uplands and rocky plains of central and southern American states such as Texas, Kansas, Colorado, Oklahoma and Iowa (Hobbs, 1989; Foster, 1991). The frequent usage of Echinacea within the Native American tribes had close relationship with the availability of *E. angustifolia* species (Hobbs, 1989; Bauer, 1998b).

1.2.1. Echinacea, a Growing Alternative Medicine

The Native Americans, especially the Plains Indians, use Echinacea externally mainly to treat poisonous insect bites, snakebites and all kinds of skin irritations and problems. Internally, Echinacea was taken to break fevers, combat sore throat, toothache, mumps or headache. It was also used to treat ailments like smallpox and measles (Foster, 1991).

From 1845 to the 1930's, there was a group of doctors, known as 'Eclectics', who used herbal medicines and helped bring Echinacea to the forefront of western herbal medicine. Among them, Dr. John King wrote the *King's America Dispensary* and promoted the use of Echinacea. As well, John Uri Lloyd started the first pharmaceutical preparations of Echinacea in the United States. Both King and Lloyd are credited for the introduction of Echinacea for medicinal purposes. Even earlier, H.C.F. Meyer had begun selling his patented medicine, 'Meyer's Blood Purifier', containing Echinacea extract, hops and wormwood, among other things (Hobbs, 1989; Foster, 1991).

In the history of many human cultures, medicinal herbs have been the primary health care for centuries until the recent development of modern medicine in the

beginning of 20th century (Bannerman *et al.*, 1983; Wills *et al.*, 2000). Even in the late 20th century, about 70% of the world population has been estimated to still use herbal medicines as their main source of remedies for ailments (Bannerman *et al.*, 1983).

In the Western countries, the improvement in medical care comes with a dramatic increase in the healthcare cost. Alternative medicines appeared to have caught the public's attention as a means to good health and well being, and a possible way to reduce this escalating cost. As early as 1982 in Germany, a survey had noted that herbal teas were used for health benefit by 76% of women. Overall, 52% of the persons surveyed used herbal remedies for minor ailments (Anonymous, 1982).

According to Grimwald and Buttel (1996), the European Union (EU) had US$6 billion sales of medicinal herbal preparations, which accounted for approximately half of the world retail market of herbal medicines. Germany contributed 45% of the sales in the EU market, while France was second with total sales of $1.8 billion. Italy was third with 11% of the market share, followed by the remaining EU countries with the combined total sales of about $840 million. In the early 1990s, there were over 280 Echinacea products and phytotherapies in the German market (Foster, 1991) and in 1998, the number of drugs containing Echinacea increased to over 800, including homeopathic preparations (Bauer, 1998b). This is also reflected in the fact that many of the studies of this American medicinal plant were carried out in Germany and the results were published in German scientific journals (Hobbs, 1989; Tyler, 1998).

Echinacea has been approved by the German Commission E for treating respiratory and urinary tract infections (Blumenthal *et al.*, 1998). But in North America,

Echinacea could not be sold as drug but it is sold as a 'supplement' in health food outlets and grocery stores (Foster, 1991).

In the United States, even though there have not been many modern scientific or clinical studies on Echinacea (Foster, 1991; Bauer, 1998b; Tyler, 1998), its products accounted for about 9% of the total value of the medicinal herb market in 1998. Retail sales of medicinal herbs in the U.S. market in 1998 were estimated to be US$4 billion, almost a 250 fold increase from the year 1994 (Brevoort, 1998). The best selling herbal supplements in health food stores in the U.S. were Echinacea products in 1996, 1997 and 1998 (Brevoort, 1996, 1998; Richman and Witkowski, 1998).

In the booming U.S. botanical market, Echinacea and other herbal medicines have been gaining more acceptance. A dozen national insurance companies are prepared to cover the cost of alternative health care, which in some cases also cover the prescriptions of botanicals (Brevoort, 1998). Using botanical medicines could tremendously save on drug costs compared with the regular pharmaceuticals, according to a survey by Kincheloe (1997).

Echinacea has steadily increased in popularity among the Americans in the category of complementary and alternative medicine (CAM) (Ness *et al.*, 1999). King (1985) noticed a similar trend during the past 20 years in Australia. Based on a survey conducted in a preoperative clinic over eleven weeks, Tsen *et al.* (2000) observed a dramatic increase in the use of CAM, where 22% of the presurgical patients reported the use of herbal remedies while women and people aged 40-60 years were noted to have a higher tendency to use herbal medicines. Again, Echinacea was the most commonly used herb.

In Europe, commercial cultivation of Echinacea is mainly in Germany, and to a smaller extent in Great Britain. Cultivation is wide spread in the United States, Australia, New Zealand and Canada (Foster, 1991; Ellyett and Morey, 1996). Even though there are some Echinacea cultivars in Canada, no statistics is available for the production and the area of cultivation.

There are different kinds of Echinacea products on the market, prepared from freeze-dried roots and aerial parts, or from the fresh plant (Bauer, 1998b). Echinacea ointments are used for wounds, sores and inflammatory skin diseases like minor burns and sunburn. Oral dosages of tinctures and extracts are for cold, flu, infections and canker sores induced by viruses. There are fast acting Echinacea products in injectible form, which are only available in Germany (Foster, 1991; Hobbs, 1994). There are also products in the form of tea made of Echinacea leaves.

1.2.2. Clinical Studies Involving Echinacea

Recent clinical research work involving Echinacea proved the effectiveness of this best selling herbal medicine. It has been found to be safe for vulnerable people such as pregnant and breast-feeding women (at moderate levels), children and the elderly (Parnham, 1996; Davis, 1999).

Barrett *et al.* (1999) carried out an extensive clinical review on the evidence of effectiveness of oral dosages of Echinacea extracts on upper respiratory infections (URIs). Their conclusion suggested that Echinacea would probably be safe and effective if taken during the early onset of illness, while there was little evidence on prolonged

usage for the prevention of URIs. This was also demonstrated by a randomised, double-blind placebo-controlled study on the efficacy of Echinacea herbal tea (Lindenmuth and Lindenmuth, 2000). Later, Manley *et al.* (1999) added further clinical evidence on its safe application to infants and children, supporting the safety of Echinacea for a wide range of population. Barrett *et al.* (1999) also commented on the wide range of composition of Echinacea preparations.

Dorn *et al.* (1997) reported on 160 patients, 83 males and 77 females aged above 18, on the URIs trials using a liquid form of *E. pallida* extracts. There was no significant difference between the sex, age and weight of the subjects correlated with the outcome when mixed factorial analysis was conducted. The specific clinical signs and symptoms were improved and the duration of illness was significantly reduced from 13 to 9.8 days for bacterial infection and from 13 to 9.1 days for viral infection (Dorn *et al.*, 1997).

On the other hand, a study involving 117 patients on experimental rhinovirus cold showed that an Echinacea preparation did not have any significant effect on the occurrence of infection or the severity of illness (Turner *et al.*, 2000). Analysis of the chemical profile of the Echinacea preparation revealed that it contained 0.16% cichoric acid with almost no echinacoside or alkylamides (Turner *et al.*, 2000). These results may provide further evidence that alkylamides might be the constituents responsible for the immunostimulatory effect.

Of the various activities attributed to Echinacea, the one that is probably the best substantiated is its immune-stimulant effect. This is brought about by three different effects: 1) stimulation of phagocytosis; 2) increasing respiratory activity and 3) causing

increased mobility of the leukocytes (Harnischfeger, 1985). However, the exact mechanisms responsible for these effects still remain unknown (Foster and Tyler, 1999).

Immunostimulants are agents that stimulate the immune system in a non-specific manner and an increase in phagocytosis (by macrophages) and granulocytes are important factors in immunostimulation. Oral dosage is as effective as parenteral dosage forms though acting more slowly (Hobbs, 1989; Bauer and Wagner, 1991; Schulz *et al.*, 1998).

Eilmes (1976) provided evidence of compounds from the roots acting as a whole against viruses. He observed that the whole extract had greater interferon-like activity than the pure chemical compounds and suggested that more chemical constitutes in Echinacea were involved in the antiviral activity. John Uri Lloyd revealed from his fourteen years experiment with Echinacea that a tincture of the dried root of *E. angustifolia*, containing 69% alcohol, which had lipophilic fraction of the roots, was the best preparation (Foster, 1991).

In a recent immunostimulatory and antioxidant activity experiment carried out by Rininger *et al.* (2000), Echinacea herb (entire plant above ground) and root powders stimulated macrophage cytokine secretion in murine *in vitro*. Echinacea preparations also enhanced the viability of human peripheral blood mononuclear cells (*in vitro*) with optimal dosage of 1 µg/mL. They further reported that Echinacea extracts obtained from various suppliers and local pharmacy and health food stores that had been standardized to 4% phenolic compounds content were not active as immunostimulant but displayed anti-inflammatory and antioxidant properties to different extents (Rininger *et al.*, 2000). They

also predicted that the immunostimulatory activity of Echinacea could survive digestion *in vivo* (Rininger *et al.*, 2000).

1.2.3. Identification and Bioactivity of Echinacea Constituents

Similar to other plants, the constituents of Echinacea range from polar compounds, such as polysaccharides and glycoproteins, to the moderately polar caffeic acid derivatives, such as echinacoside and cynarine, and to the lipophilic alkylamides and polyacetylenes (Bone, 1997; Bauer, 1998b).

Wagner *et al.* (1984) isolated immunostimulatory polysaccharides from *E. purpurea* and *E. angustifolia* with molecular weights ranging from 25,000 to 50,000 and higher, using granulocytes and carbon-clearance tests. Stimpel *et al.* (1984) purified the polysaccharides in the aqueous extracts of *E. purpurea* culture and found that it possessed immunostimulatory properties. Polysaccharides had been found to strongly activate macrophages by developing high cytotoxicity against the tumor cells (Stimpel *et al.*, 1984). It also stimulated the production and secretion of oxygen radicals of macrophages. However, its function did not have a direct relationship with T lymphocytes (Stimpel *et al.*, 1984).

Purified polysaccharides from a large cell culture of *E. purpurea* could increase the activity of polymorphonuclear cells so that the ability to kill yeast and bacteria was significantly enhanced (Roesler *et al.*, 1991). Even a low dosage (i.v. injection) of 5 mg of purified polysaccharides from cell culture of *E. purpurea*, given at 0.05-0.07 mg/kg body weight, could induce surprisingly strong *in vivo* effects in humans (Roesler *et al.*,

1991). Same source of polysaccharides also restored the resistance in mice against infection of *Listeria monocytogenes* and *Candida albicans* in an infection stress test (Steinmüller *et al.*, 1993). Polysaccharides from *E. angustifolia* are considered to be the main principals for anti-inflammatory activity (Tubaro *et al.*, 1987).

Alcoholic extracts of Echinacea, either aerial or root parts, contain caffeic acid derivatives and lipophilic polyacetylene-derived compounds and essential oils (Bauer, 1998b). Echinacoside is a water-soluble crystalline compound from *E. angustifolia* and was found to possess slight antibiotic activity against streptococci and *Staphylococcus aureus* (Stroll *et al.*, 1950). They also reported about 1% (w/w) echinacoside in the root, which was consistent with the results of Bauer and Wagner (1991), claiming 0.3-1.3% (on dry weight basis) echinacoside in the roots of *E. angustifolia*. Two-year-old roots of *E. purpurea* were found to contain 1.8% (w/w) echinacoside (Ooraikul *et al.*, 2000), which contradicts some other findings (Bauer and Wagner, 1991; Bauer, 1998b).

Cichoric acid (2,3-o-di-caffeoyl tartaric acid) has been shown to play an important role in the biological activity of Echinacea extracts. It has inhibitory effect on hyaluronidase and can also stimulate phagocytosis activity *in vitro* and *in vivo* (Soicke *et al.*, 1988; Facino *et al.*, 1993).

Bauer *et al.* (1988a) had identified cynarine in Echinacea roots and found it to be unique to *E. angustifolia* species. Cynarine was believed to be the active constituent in Globe Artichoke (*Cynara scolymus* L.), which belongs to the same family of *Asteraceae* as Echinacea. Extracts of this plant containing cynarine was used for the treatment of jaundice caused by malfunction of the liver in the Middle Ages (Panizzi *et al.*, 1954). Caffeic acid derivatives in Echinacea species were found to have a protective effect on

the free radical-induced degradation of type III collagen of skin and their inhibition potential was in the order of echinacoside ≈ cichoric acid > cynarine (Facino *et al.*, 1995).

Essential oil is present in Echinacea species. The major essential oil components are borneol, alpha-pinene and sespuiterpene derivatives (Bauer and Wagner, 1991). The essential oil content of roots varies among different species, ranging from 0.1% in *E. angustifolia* (Bomme *et al.*, 1992a, b) to 2.3% (w/w) in *E. pallida* (Neugenbauer, 1949).

Hexane extracts of Echinacea, containing isobutylamides and polyactylenes, were reported to inhibit the growth of yeast strains under near UV irradiation and, to a lower extent, without irradiation (Binns *et al.*, 2000). Although alkylamides do not commonly occur in plants, they are important components in the lipophilic fraction of Echinacea extracts and are the chemicals that are responsible for the tingling and numbing sensation on the tongue if the plant is chewed (Bauer and Wagner, 1991; Bone, 1997). Alkylamides are consti-tuents contributing to the immune-stimulating effect of Echinacea species. They are composed of a highly unsaturated carboxylic acid (often with double or triple bonds) and an amine compound, either isobutylamine or 2-methylbutylamine. There are a total of 10 and 15 alkylamides identified in *E. purpurea* and *E. angustifolia*, respectively, six of which exist in both species (Bauer *et al.*, 1988b, 1989b).

Echinacea was also evaluated as a natural insecticide (Jacobson, 1954). Jacobson isolated echinacein, an isobutylamide, as the insecticidal component in the roots. Echinacein was found to be effective against German cockroach, adult houseflies and the yellow mealworms (Jacobson, 1967). In 1975, an insect-growth regulator, echinolone was isolated, which would mimic juvenile hormones in the yellow mealworm (Jacobson *et al.*, 1975).

In the search for immunostimulant compounds in Echinacea, echinacoside was once regarded as the principal compound; however, it is considered as a component of minor importance (Hobbs, 1989; Bauer and Wagner, 1991). Lipophilic fractions of *E. angustifolia* and *E. pallida* are more active than hydrophilic fractions, while *E. purpurea* stimulated phagocytosis by 40% after the enhancement of cichoric acid (Bauer *et al.*, 1989a).

Bauer *et al.* (1989a) had shown that lipophilic fractions of the extracts from the three species all had stronger immunological activities (carbon elimination test and stimulation of phagocytosis) than the hydrophilic fractions. Of the three species tested, positive effects were observed only in the hydrophilic fractions of *E. purpurea*. Therefore, Bauer *et al.* (1989a) suggested that isobutylamides and/or polyacetylenes of the essential oils, which could be found in the lipophilic fractions, may be the active components of Echinacea responsible for immunostimulation.

1.2.4. Analysis of Caffeic Acid Derivatives and Alkylamides

High performance liquid chromatography (HPLC) has been successfully utilised in the analysis of caffeic acid derivatives and alkylamides in Echinacea using different mobile phases for polar and lipophilic components (Bauer *et al.*, 1988a). Bauer and Foster (1991) applied the same analytical conditions and examined the constituents of two less commonly used Echinacea species, *E. simulata* and *E. paradoxa*. Glowniak *et al.* (1996) modified the mobile phase of HPLC used by Bauer *et al.* (1988a) and obtained

distinctive chromatograms for six species, including *E. purpurea*, *E. angustifolia*, *E. montana*, *E. multiflora*, *E. commutata* and *E. umbellata*.

Other methods have been developed in recent years for the analysis of caffeic acid derivatives. Facino *et al.* (1993) established fast atom bombardment tandem mass spectrometry (negative ion) (TAB-MS/MS) method and characterized caffeic acid derivatives in *E. angustifolia* roots without reporting echinacoside. Micellar electrokinetic chromatography (MEKC) was also utilized to define the fingerprints of caffeic acid derivatives in Echinacea, both in roots and aerial parts, and indicated that MEKC was a valuable alternative method for quality control (Pietta *et al.*, 1998).

Bauer *et al.* (1988b) isolated four isobutylamides and one methylbutylamide from the roots of *E. purpurea* in addition to the existing lipophilic constituents identified by Bohlmann and Grenz (1966), Bohlmann and Haffmann (1983) and Martin and Becker (1985). Therefore, there are altogether 10 alkylamides in *E. purpurea*: undeca-2E,4Z-dien-8,10-diynoic acid isobutylamide, undeca-2Z,4E-dien-8,10-diynoic acid isobutylamide, dodeca-2E,4Z-dien-8,10-diynoic acid isobutylamide, dodeca-2E,4E,10E-trien-8-ynoic acid isobutylamide, dodeca-2E,4E,8Z-trienoic acid isobutylamide, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide, trideca-2E,7Z-dien-10,12-diynoic acid isobutylamide, undeca-2E,4Z-dien-8,10-diynoic acid 2-methylbutylamide and dodeca-2E,4Z-dien-8,10-diynoic acid 2-methylbutylamide (Bauer *et al.*, 1988b).

Shortly afterwards, Bauer *et al.* (1989b) reported the isolation and structures of 15 alkylamides from *E. angustifolia* by soxhlet extraction, with n-hexane as solvent, similar to their protocol applied to *E. purpurea*. Four compounds had previously been identified by Verelis (1978), Bohlman and Hoffmann (1983) and Martin and Becker (1985). Six of

the compounds were isolated for the first time and the remaining compounds also existed in *E. purpurea*. These two species have six compounds in common, with the same two isomers, dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide as the main constituents.

Bauer *et al.* (1988a) studied the thin-layer chromatography (TLC) and HPLC methods for the differentiation of *E. pallida* and *E. angustifolia* roots since these two species had been misidentified in the past. Obtaining root samples from USA, Germany and Switzerland, they further reported the chemical constituents of *E. pallida*. Alkyl ketones were the typical constituents of *E. pallida* while alkylamides were usually absent. Comparison of the phenolic composition of these two species indicated that the identification of cynarine may prove to be useful for their differentiation since cynarine exists only in *E. angustifolia*. The two species have similar echinacoside content of 0.5-1.0% (w/w) in dried roots.

The three commonly used Echinacea species have different alkylamide contents in the roots and aerial parts as detected by TLC and HPLC. In roots, *E. angustifolia* and *E. purpurea* had different main moieties, which exhibited different patterns in HPLC (Bauer and Remiger, 1989). There were no characteristic differences between the three species in the aerial parts (Bauer and Remiger, 1989). The highest content of main alkylamides was found to be mainly in *E. angustifolia* roots (Bauer and Wagner, 1991). The authors reported that there was no echinacein present as reported by Jacobson (1967).

More recently, several new methods were developed for the analysis of alkylamides in the three Echinacea species. He *et al.* (1998) applied HPLC-electrospray

mass spectrometry (HPLC-ES-MS) for the analysis of alkylamides in the *E. purpurea* roots and aerial parts and reported a possible new compound. Aerial parts of *E. angustifolia* and *E. pallida* were analysed by HPLC-ES-MS.

A gas chromatography – mass spectrophotometry (GC-MS) method for alkylamides was developed by Lienert *et al.* (1998) and was used both for the identification and quantification, even without the use of any pure alkylamide standards. Three different types of gas chromatograms were obtained for the three species of Echinacea using the same method, and they were different enough to differentiate these three species. There was no significant difference among the different extraction methods used prior to GC-MS analysis, e.g. soxhlet extraction, supercritical fluid extraction (SFE) and maceration with three different solvents (Lienert *et al.*, 1998).

HPLC is more commonly used for the analysis of both phenolic compounds and alkylamides. For example, HPLC was used to determine alkylamide and/or cichoric acid content in different parts of Echinacea (Perry *et al.*, 1997); in *E. purpurea* grown in Australia (Rogers *et al.*, 1998; Wills and Stuart, 1999); during plant growth (Stuart and Wills, 2000); and in the study of the processing, drying and storage effects (Perry *et al.*, 2000).

1.3. EXTRACTION PROCESS

1.3.1. Principles of Extraction

1.3.1.1. Extraction Solvents

Plant materials, like Echinacea have a cellular structure and the active components are usually found inside the cells. They have to be leached out and concentrated before use as herbal extract. Liquid-solid extraction process, also called leaching or washing, is the most common process adopted for the commercial production of the extracts. Sugar beets are washed with hot water containing lime; vanilla beans are washed with aqueous ethanol (35% ethanol) and soybean seeds are extracted with hexane. These are just a few examples of the extraction processes employed in the food industry (Treybal, 1980).

Solvents used in the extraction process are liquids under the extraction conditions in which solutes can dissolve and be recovered unchanged upon solvent removal. Many substances conform to this definition, water being the most abundant. Materials that could be liquefied under extreme temperature and pressure conditions are not suitable for application purposes. On the other hand, some 'permanent' gases, like nitrogen and carbon dioxide, have been used as 'supercritical solvents' and have attracted increasing attention in recent years (Marcus, 1998).

1.3.1.2. Basic Principles and Parameters of Extraction

The driving force of an extraction process, controlling the diffusion of the solute from solid to solvent, is its concentration difference between the solid matrix and the solvent (Treybal, 1980; Lydersen, 1983). Diffusion in solids is much slower compared with that in gases and liquids and is usually the governing factor in food and pharmaceutical extractions (Lydersen, 1983). Therefore, any change in the solid or solvent promoting the diffusion rate of the solute from solid to solvent will increase the efficiency of the solvent extraction process.

Appropriate preparation of the solid material is the first step for a successful solvent extraction process. High quality of starting material is always the primary requirement for a good end product for foods and phytotherapies. Hobbs (1994) stated that the form of Echinacea products is of less importance than the quality of the starting materials.

Drying of plant roots, stems and leaves makes the solute more concentrated than that in fresh ones. Crushing and grinding of these cellular structured plant materials create increased surface area for the solvent to interact with the solid and make the solute more accessible to the solvent, hence, greatly accelerating the extraction process. However, it is impractical and sometimes unacceptable to grind the solid into very fine particles because the heat generated during grinding may cause degradation of the solute and fine particles may prevent the solvent from flowing freely (Treybal, 1980; Geankoplis, 1983; Lydersen, 1983).

Increasing the solvent temperature is also desirable for the extraction process. High temperature leads to higher solubility of the solute in the solvent as well as higher

diffusivity and lower viscosity of the solvent, resulting in higher extraction rate and shorter extraction time. The highest attainable temperature is normally limited by the boiling point of the solvent, and in some cases, high temperature may cause the loss of valuable volatiles and degradation of the thermally unstable components (Treybal, 1980; Lydersen, 1983).

Available volume of the solvent to dissolve the solute (solvent to feed ratio) is another important factor. Ideally, the more solvent the better but this is not always practical in industrial scale operations. The least yet sufficient volume of the solvent needed for a given amount of solids has to be determined from experiments in consideration of economical production.

Extraction time is also an important factor. During the initial stage of the extraction process, the concentration (g/g) of the solute in the solvent is constant and limited by its solubility. At this stage, the rate-limiting step (or the largest mass-transfer resistance) is the actual transfer of the solute from the surface of a solid particle to the solvent. Extraction rate slows down after some time and the diffusion rate of the solute from inside the solid to the surface of the solid becomes the limiting factor. The concentration of the solute in the solvent is dramatically decreased as the extraction reached diffusion limiting stage. Therefore, sufficient extraction time should be allowed for the maximum recovery of the targeted solute from the solids.

Selection of an appropriate solvent involves consideration of many factors including solubility behavior of the solute of interest, possible chemical interaction between the solvent and the solute, its miscibility with water, and physical properties of the solvent, e.g. density, viscosity and volatility. Other criteria are its availability, cost,

toxicity, hazardousness and environmental acceptability (Marcus, 1998). Therefore, selection of the solvent and finding the optimal extraction conditions is always a process of compromise, considering many factors involved.

Extraction of various botanical materials has been investigated. For example, hot water has been used for the extraction of *Salvia miltiorrhiza* roots (Nan *et al.*, 2000); methanol or ethanol and their aqueous solutions have been studied for the extraction of alkylamides and phenolic compounds in Echinacea (Rogers *et al.*, 1998; Bergeron *et al.*, 2000; Stuart and Wills, 2000) and n-hexane was used for the extraction and isolation of hyperforin from *Hypericum perforatum* (Orth *et al.*, 2000). These are a few examples of the extraction process for botanical materials.

1.3.2. Ethanol Extraction

1.3.2.1. History, Properties and Toxicology of Ethanol

Alcohols are a group of compounds that are important in scientific fields and industries and may be known under various names. Considering ethanol (CH_3CH_2OH), it is known as ethyl alcohol, ethyl hydroxide, grain alcohol, spirits of wine, fermentation alcohol, etc. Each name may be related to either some specific industry or the origin of ethanol (Monick, 1968).

Knowledge of ethanol has a close relationship to the knowledge of fermentation, dating back about 6000 years. Fermentation scenes were found on Mesopotamian pottery from about 4200 B.C. (Monick, 1968). During ancient times, man discovered that the juice of fruits and berries changed substantially when aged for a short period of time.

This was mysterious and happened beyond their explanation. The Egyptians, Romans and Hindus worshipped their own gods who were in charge of wine and alcoholic drinks. Even though it was produced only in crude form with little understanding of the scientific principles behind it, ethanol is no doubt one of the oldest known organic chemicals (Monick, 1968).

Ethanol was regarded as the magic 'spirit' of wine and all ancient references discussed it as a beverage. The Arabians, who learned from the Egyptians, established the distillation of ethyl alcohol and hid it from Europeans until the 12^{th} or 13^{th} century, A.D. (Monick, 1968).

The chemistry of ethanol was not studied until approximately the 19^{th} century although the distillation of ethanol was known to various degrees to the experts. Wood spirit, methanol, was once confused with ethyl alcohol after its discovery and their formulas were disclosed in 1836 (Monick, 1968).

Chemically, ethanol is a typical n-saturated monohydric alcohol, especially in the reactions concerning the hydroxyl group, and belongs to protic and protogenic solvents in the common solvent classification scheme (Marcus, 1998). It is a colourless and neutral liquid with a molecular weight of 46.07. It can be mixed with water at any proportion where the mixing process results in a volume contraction and produces heat. Aqueous ethanol solution and the properties of this solution are of importance for the analysis of simple mixtures (Monick, 1968).

Ethyl alcohol can also be mixed with most other organic solvents to form azeotrope systems. An azeotrope is a mixture of two or more liquids in the form of loosely held molecular compounds that have a fixed boiling point, which is generally

lower than that of the individual liquids. The formation of azeotropes containing ethanol has important commercial application in the production of anhydrous alcohol (Monick, 1968).

Ethanol is an excellent solvent for many compounds: resins, fats and oils, fatty acids, alkali hydroxides, hydrocarbons, some inorganic salts, and numerous organic compounds (Monick, 1968).

Enjoying a wide application in industry, practical experience reveals that ethanol is moderately safe in industries. It has a lower chronic toxicity than methanol and does not form toxic metabolites. Proper ventilation is essential when working with ethanol, however, prolonged inhalation of its vapour will result in the irritation of the mucous membranes of the eye and respiratory tract, headache, nervousness, dizziness, tremors, fatigue, nausea and narcosis. These toxic effects from inhalation, however, have been rarely recorded (Monick, 1968).

1.3.2.2. Production and Utilization

The earliest manufacture of ethanol primarily utilized natural products (carbohydrates) and a natural process (fermentation) where making alcohol was regarded as a personal activity. However, in the 20th century, synthetic production became the prominent source of ethanol. It was achieved by the availability of large volumes of by-products from petroleum refining (Monick, 1968; Treybal, 1980).

There are unique requirements for the definitions and conversion factors to different industrial fields. Industrial alcohol is a mixture of ethanol and water (usually 95% ethanol and 5% water, by volume) combined with other chemical products, which

make it unfit for beverage use. On the other hand, pure ethanol finds special applications in the fields of pharmaceutical, cosmetics, flavouring extracts and food industries. Approved education, scientific, and medical organizations and public agencies could obtain pure ethanol tax-free following a special procedure (Monick, 1968).

Ethanol has been mainly used in the synthetic rubber industry, chemical industry, like production of acetaldehyde, acetic acid, ethers, and pharmaceutical products; in the paint and lacquer industry, as an anti-freeze agent; in the plastic and synthetic resin industry and in the explosives industry (Browing, 1965).

The choice of solvent in medicinal herbs, as well as in foods, must present the maximum selectivity, have the best capacity of extraction and be compatible with the properties of the material to be extracted. According to the pharmarcopeias, ethanol is the solvent of choice for obtaining classic extracts such as tinctures and soft, fluid and dry extracts; and it is still widely used in pharmaceutical formulations (Bombardelli, 1991).

Medicinal spirit is a form of phytomedicine, which is a solution of a volatile substance dissolved in alcohol or in aqueous alcohol. Tincture is another form, prepared from alcoholic or hydroalcoholic solutions from botanicals (Schulz *et al.*, 1998). Lloyd Brothers Pharmacists Inc., which was formed by John Uri Lloyd and brothers, manufactured a tincture Echinacea product and was the first pharmaceutical production of Echinacea (Foster, 1991). For centuries, ethanol has played an important role in the medical sector as an antiseptic for wounds, skin diseases etc. That is why pharmaceutical industry uses ethanol for the production of drugs and disinfectants.

Sauvesty and Page (1992) found that ethanol caused the least changes in phenolic acids of sugar maple leaves and water resulted in the greatest loss. There was no

significant difference between methanol and ethanol at 50% and 80% (v/v) for the recovery of phenolic compounds. Aqueous ethanol (50% ethanol, v/v) was selected for its low cost and low toxicity and it proved to be a reliable solution for the extraction of those components from sugar maple leaves (Sauvesty and Page, 1992).

1.3.3. Supercritical Fluid Extraction

For over 90 years, solvent extraction and its variants have been the classical liquid-solid separation methods in analytical and food industry (Hawthorne, 1990). The liquid extraction process is widely used in oil refining, flavor, hops and food additives production. Accompanying these techniques has been an excessive usage of organic solvents as extractives or partitioning phases. Thermal degradation of spices and fragrances can also occur during these processes.

There is a large amount of cleanup work that needs to be done following solvent extraction and requires great amount of energy to separate the solvent and perspective products. There is always a certain amount of organic solvent left after the removal of the solvent from the final products. Growing concerns regarding the toxicity of the commonly used solvents, the storage and disposal costs, environmental dangers of the solvents and the emission of hazardous solvents into the air during concentration have led to the development of alternative extraction methods (Hawthorne, 1990; Salisbury *et al.*, 1992).

Beginning in 1990, regulatory legislation, such as the U.S. Environmental Protection Agency's (EPA) Pollution Prevention Acts; the Superfund Amendments and

Reauthorizaion Act (SARA); the Resource, Conservation and Recovery Act (RCRA); and the Montreal Protocols have focused on the reduction of the use of specific organic solvents that are harmful to the environment (Salisbury *et al.*, 1992). No doubt these pieces of legislation constrain the reinvestment in scaling up the present production or building up new plants employing solvent extraction methods. Nonetheless, ethanol is the choice solvent in the pharmaceutical industry (Bombardelli, 1991).

Supercritical fluid extraction (SFE) has gained more acceptance as the industries are looking for alternative solvents and extraction processes, which can be easily operated with minimal operation steps, have rapid extraction rates and are environmentally friendly. SFE process offers considerable advantages, such as better recovery of volatile components and cleaner extracts than liquid solvent extractions, which frequently contain large amounts of organic solvent that need to be removed. Obtaining cleaner extracts from SFE reduces the need for cleanup and facilitates the quantification of target components (Bevan and Marshall, 1994; Berglöf *et al.*, 1999; Lang and Wai, 2001).

1.3.3.1.Historical Review of Supercritical Fluid Extraction

Supercritical phenomena and the solvation potential of a supercritical fluid (SF) are not new. As early as in 1869, Andrews (1875-1876) had discovered the critical phenomenon and described his experimental apparatus and the observation of properties of critical carbon dioxide. The critical point of carbon dioxide he discovered, 30.92°C and 7.4 MPa are very close to the values that are presently accepted, which is 31.1°C and 7.38 MPa, respectively (Brunner, 1994; McHugh and Krukonis, 1994).

Hannay and Hogarth (1879) were credited for their discovery of the ability of a SF to dissolve solid materials. They found several inorganic salts dissolved in ethanol by increasing the pressure of the system and decreasing the pressure caused the salts to precipitate out from ethanol at a temperature above its critical temperature (240.8°C). Substantial discussion and debate between Ramsay (1880) and Hannay and Hogarth were initiated about the solubility of salts in SF. As a consequence, this debate attracted more attention of scientists to be engaged in the experimental and theoretical investigations involving supercritical phenomena (McHugh and Krukonis, 1994).

After Hannay and Hogarth (1879), a number of reports explored the solubility phenomena for a wide range of materials. McHugh and Krukonis (1994) addressed the history in more detail and provided a review of developments over the past century.

1.3.3.2. Basic Principles of Supercritical Fluids

A supercritical fluid is defined as a compound in the state above its critical pressure (P_c) and critical temperature (T_c). The highest temperature at which a gas can be converted to a liquid by increasing the pressure is called the critical temperature. The highest pressure at which a liquid can be converted to a traditional gas by an increase in temperature is called the critical pressure. This single-phase compound at conditions above the critical point with unique physico-chemical properties and high density is referred to as the supercritical fluid. It is highly compressible but cannot be liquefied. The properties of the substances in this region are neither a true liquid nor a true gas.

For a pure substance, the shaded area in the pressure-temperature (P-T) diagram as presented in Figure 1.1 shows the critical region. Substances in the supercritical

region have useful properties for extraction processes. Generally, the solvating power of a supercritical fluid increases as its density increases (Bevan and Marshall, 1994; McHugh and Krukonis, 1994).

A number of solvents have been explored for their solubility phenomena under supercritical conditions, such as carbon dioxide, nitrous oxide and light hydrocarbons. Figure 1.2 shows the reduced pressure-density isotherms of supercritical and subcritical carbon dioxide. At a temperature below the critical temperature, for example $0.96T_c$, isothermal compression of gas carbon dioxide from point A to B results in the appearance of liquid phase, which is corresponding to point C, where point B and C are in equilibrium. When further condensed at constant saturation vapour pressure, the gas CO_2 turns to liquid CO_2 completely. If compressed further at constant temperature, the liquid CO_2 gives the isothermal density curve of CDE (Fig. 1.2).

Above the critical temperature (31.1°C) (reduced temperature, $T_r = T/T_c = 1.0$), large changes in density can be seen as the pressure is varied. CO_2 fluid with a density similar to that of a liquid is attainable at pressures not much in excess of the critical pressure or when temperature is close to T_c. At more elevated temperatures, such as $1.6T_c$, much higher pressure is needed to obtain the same density, compared to what is needed at a lower temperature like $1.06T_c$. Therefore, supercritical extraction is conducted at temperatures less than 100°C above T_c (Williams, 1981).

It is readily accepted that liquid carbon dioxide (like CDE in Fig. 1.2) could act as a solvent. This can be explained in terms of the intermolecular forces arising from the close packing of solvent molecules around the solute molecule, and thus could be related

to the density of the liquid. Therefore, supercritical carbon dioxide, having a similar density as liquid carbon dioxide could also act as a solvent (Williams, 1981).

Figure 1.2 shows that at a given pressure, the solvent power of the supercritical fluid, i.e. the density, is greater when it is at a lower temperature; and at a given temperature, the solvent power of the supercritical fluid is greater at higher pressures. In other words, if a supercritical solution is decompressed, the solvent power of the gas becomes very small and thus the dissolved material would be separated, allowing the recycle of the recovered gas.

Supercritical fluids exhibit physical properties intermediate between those of liquids and gases and offer a convenient way to achieve solvating property, which have gas- and liquid-like characteristics without actually changing its chemical structure. That means supercritical fluids can be considered as a continuously adjustable solvent. Table 1.1 illustrates how supercritical fluids (SFs) compare to gases and liquids in terms of their physico-chemical properties.

The density affects solvating power of a solvent: the higher the density, the greater the solvent strength. SFs have as high a density as that of liquids. Both liquids and SFs have high solvent strengths and can dissolve different types of solutes.

SFs also have a diffusion coefficient close to that of gases but 10-100 times higher than that of liquids (Table 1.1). High diffusion rates enable SFs to transport dissolved solutes through a matrix rapidly. SFs have relatively low viscosity, like gases, and enable them to penetrate a given matrix much easier than a liquid. Diffusivity increases with an increase in temperature at fixed pressure, whereas viscosity decreases with a temperature increase (Brunner, 1994).

These combinations of physico-chemical properties suggest that SFs can penetrate a given matrix as if it was a gas under high pressure but had solvating power of a liquid, where its solvation properties vary greatly with temperature and pressure (Williams, 1981; Palmieri, 1988; Bevan and Marshall, 1994).

Increasing the temperature at a given density increases the diffusion coefficient in the supercritical fluid as well as the volatilization of the solute. Within the solubility of the solute in the fluid, SFE recovery of solutes can be greatly enhanced by increasing temperature of extraction at a constant fluid density, whereas there is a report stating that extraction temperature has less influence than density on the recovery on analytical scale (Berglöf *et al.*, 1999), which also depends on solute properties.

Importantly, conditions do not exist where a supercritical fluid can have both the solvation capacity of the liquid-like phase and the high diffusion rate of the gas-like phase at the same time (Smith, 1999). However, optimal combination of pressure and temperature exists for a compound of interest to be extracted from a matrix. In addition, the effect of other variables, listed in Table 1.2 should also be considered and combined with the well-established density and temperature effects.

1.3.3.3. Choice of Supercritical Extraction Solvents

Solvation of a solute by a supercritical fluid is a general phenomenon exhibited by all supercritical solvent-solute pairs as long as the solute is not infinitely miscible with the solvent. Table 1.3 lists the critical temperature and pressure for a number of gases and liquids. A careful inspection of this table reveals the following trends:

1) Most hydrocarbons have a critical pressure close to 5 MPa;

2) The critical temperatures for light hydrocarbons, such as ethylene and ethane are around room temperature; cyclic aliphatics and aromatics have higher critical temperatures;

3) Carbon dioxide has a mild critical temperature and a slightly elevated critical pressure;

4) Ammonia and water have high critical temperatures and pressures, which is a result of polarity and hydrogen bonding;

5) Non-polar materials have relatively low critical parameters (e.g. CO_2, T_c = 31.1°C, P_c = 7.38 MPa), whereas polar compounds have high critical parameters (e.g. H_2O, T_c = 374.2°C, P_c = 22.12 MPa) (Brunner, 1994; McHugh and Krukonis, 1994).

Because of the unique characteristics of supercritical fluids, a wide range of compounds, whose critical points are attainable under reasonable conditions, have been examined as SFE solvents. In practice, there are several reasons to choose a supercritical fluid over another solvent system. Generally, it is governed by the unique solvation and favourable mass transport properties, the ease with which the solute's extraction potential can be varied simply by the adjustment of the system pressure and/or temperature (Smith, 1999).

An ideal solvent for effective SFE should have mild critical parameters, and that is why most polar substances have not been given serious consideration for SFE. This ideal material should also be relatively inert, inexpensive, highly pure and non-toxic. Carbon dioxide best accommodates all these requirements for its low cost, availability, safety and high purity. Alternative materials (e.g. hydrocarbons, nitrous oxide) have been given limited consideration as supercritical solvents since some of these compounds exhibit no greater solvating power than CO_2, or some of them need very careful

treatment. Carbon dioxide has been the standard gas for almost all commercial systems and is probably the only realistic solvent for use in most laboratories and industries, especially the food industry (Smith, 1999).

1.3.3.4. Entrainers, Extraction Equipment and Extraction Mode

Supercritical carbon dioxide ($SC\text{-}CO_2$) is a powerful solvent for non-polar compounds but inefficient for polar compounds. In many cases, the solvating power of supercritical CO_2 at high density is insufficient to extract a solute because the solute is either not soluble or is strongly bound to the matrix.

An additional component, called entrainer, modifier or co-solvent (Ely and Baker, 1983; Brunner, 1994) is added to supercritical fluids with the intention to increase the polarity of the $SC\text{-}CO_2$ and obtain solvation ability that none of the pure components has alone. The presence of the entrainer not only enhances the solvent power of the $SC\text{-}CO_2$, but also enhances temperature and pressure dependence of solvent power (Brunner, 1994).

Employment of an entrainer is a good means for designing a large-scale separation process, which can be operated continuously and economically at constant pressure. An entrainer usually has a volatility intermediate between those of the substances to be separated and the $SC\text{-}CO_2$ (Ely and Baker, 1983).

A more popular method for increasing the solvating power is to employ a polar entrainer with $SC\text{-}CO_2$. Methanol is the most commonly used entrainer in analytical scale because it is effective in increasing the polarity. Methanol can dissolve in $SC\text{-}CO_2$ up to 20% so as to dissolve the solute from plant matrix (Lang and Wai, 2001). Water, as

a more polar entrainer than methanol, often acts as a barrier in the extraction process (Leohtay, 1997). Although ethanol is not as polar as water and methanol, it is the most appropriate entrainer for the extraction of natural products at industrial scale.

Pure SC-CO_2 serves as the fluid of choice for the majority of extraction systems. The basic components required for performing SFE consist of CO_2, a compressor/pump, heated zone/oven, extraction vessel, expansion valve and a separator/collector. The supercritical fluid extraction can be accomplished by using either static, dynamic, or a combination of static and dynamic modes (Hedrick *et al.*, 1992; McHugh and Krukonis, 1994). In industrial scale operation, the CO_2 is usually collected and recycled whereas analytical and laboratory scale units do not have such capability.

A static mode refers to the operation that allows a fixed amount of SC-CO_2 to interact with the matrix and it may be applied at the beginning of an extraction process. Dynamic extraction employs fresh supercritical fluid, which is continuously passed through the sample matrix and it is more exhaustive than the static one because fresh supercritical fluid is always in contact with the sample. Combination of an initial static period followed by a dynamic one in order to move the solute to the trap is gaining popularity, especially for situations where the solute must diffuse to the matrix surface in order to be extracted (Dean *et al.*, 1998; Lienert *et al.*, 1998).

1.3.3.5. Applications in the Food Industry

A large amount of experimental data has been accumulated on the solubility and extractability of natural products, such as steroids, alkaloids and caffeine from coffee beans, in various SF solvents such as CO_2, ethane, ethylene and NO_2. Applications of

SFE in natural products and foods have covered decaffeination of coffee and tea; deodorization of fats and oils; nicotine from tobacco; vegetable oil from seeds; food colouring from plant materials; flavours, fragrances, aromas and perfumes; hops and spices (Hoyer, 1985; Bevan and Marshall, 1994). Still, carbon dioxide is the most widely investigated solvent. Supercritical carbon dioxide, however, is not always the most effective solvent for some ingredients, like carotenoids and tocopherols in red pepper oil and some natural antioxidant extracts in aromatic herbs (Dapkevicius *et al.*, 1998; Illés *et al.*, 1999).

As early as in the 1980s, Europe had built supercritical fluid extraction plants capable of processing of millions of kg material annually (McHugh and Krukonis, 1994). A plant for decaffeinating coffee beans was built in Bremen, Germany and several plants for extracting hops and spices were built in Germany, France, and the United Kingdom. There have been between 30 to 40 supercritical processing plants operating worldwide up to 1996 where the USA and Germany have been prominent (Anonymous, 1996). The number of patents issued on various applications has increased steadily over the years.

In the decaffeination process, moist CO_2 is employed to extract caffeine from green coffee beans where dry CO_2 cannot be used either for green beans or roasted beans. It is generally thought that the caffeine is chemically bound in a chlorogenic acid structure present in the coffee bean. Thus, water somehow acts as a chemical agent freeing caffeine from its bound form in the coffee matrix (McHugh and Krukonis, 1994).

Even though there are extra steps to cleanup the CO_2 before recycle, the SC-CO_2 extraction of coffee has proven to be sound and innovative and has been a successful process. It is the first example of SFE process that has reached the commercial

processing level. General Food's Maxwell House Division (Houston, TX) owns the largest commercial supercritical CO_2 decaffeination plant in the world (Mermelstein, 1999).

There are four main producers in Europe, namely Universal Flavours Ltd. (UK), CAL Pfizer (France), Flavex GmbH (Germany) and Givaudan Roure (Switzerland). They have employed both subcritical and supercritical carbon dioxide, providing the market with a total of 51 kinds of food flavorings (Anonymous, 1996).

Even though supercritical fluid extraction process is more capital intensive upon setting up, it is always more cost effective at higher volume throughput and has a substantially lower operation cost compared to conventional solvent extraction (Mermelstein, 1999). Because CO_2 is not a hazardous solvent and there are no solvent residues in the final product, regulations are less restrictive. Supercritical fluid extraction is a powerful extraction method as it is a bonus to both ends: the consumers get quality products without paying too much in processing cost, and the manufacturers get lower cost and high quality products (Moyler, 1993a; Mermelstein, 1999).

1.3.3.6. Supercritical Fluid Extraction of Herbal Plants

Other than the SFE applications in the food industry, pharmaceuticals and nutraceuticals are the most active and recent development areas of research in SFE processing (Mermelstein, 1999).

Compounds having biological activities usually exist in herbal plants at low concentrations (Lang and Wai, 2001). SFE has provided an efficient technique for the recovery of these valuable components compared with traditional extraction methods.

Dean *et al.* (1998) have successfully extracted magnolol, the active compound from *Magnolia officinalis* medicinal plant, using SC-CO_2 without entrainer at 40°C and 24.5 MPa for 60 min, at a flow rate of 2 mL/min. The yield of magnolol from SFE was 1.34% (g magnolol/ g feed material) compared with 1.01%, the highest yield from phytosol solvent extraction. By adding 10% (v/v) of methanol and increasing the extraction temperature to 60°C while maintaining the same extraction pressure and flow rate, the yield of magnolol reached 1.86% (g/g), which was the highest recovery obtained by Dean *et al.* (1998).

Lopez-Avila and Benedicto (1997) studied the yield of kava lactones in *Piper methysticum* at SFE conditions of increased extraction pressure at constant temperature; they also added ethanol at the end of the extraction process. They had performed the extraction with SC-CO_2 alone in the beginning at 25.3 MPa and 60°C for 60 min and recovered 21.5% of total content of kava lactones. Without altering the extraction temperature during the SFE process, pressure was increased to 35.4 MPa and the cumulative recovery was 57.5% after extracting for 30 min. Further increasing pressure to 45.6 MPa and extracting for 60 min recovered 68.9% of kava lactones and the recovery rate slowed down, even with increased density of SC-CO_2 fluid. Ethanol was added at this stage and the pressure was adjusted back to 25.3 MPa and 96.4% of kava lactones were recovered after a 60 min extraction. This recovery was higher than that from methylene chloride sonication extraction.

In both cases discussed above, SFE resulted in higher extract yields than that obtained with conventional extraction, and the addition of an entrainer (methanol or ethanol) enhanced the yield further. Like SC-CO_2 is not always the most effective

extraction solvent, adding entrainer is not always effective in increasing the extraction efficiency (Weathers *et al.*, 1999).

The average molecular weight of the 15 alkylamides (assuming they have equal proportion in the root) isolated from *E. angustifolia* is 249.93 where the molecular weight of the main constituent tetraenes is 247. Since SC-CO_2 is able to extract organic components from plant materials, the lipophilic constituents, like alkylamides with low molecular weight (< 300) in Echinacea roots, are also extractable (Moyler, 1993a,b). However, SC-CO_2 extraction of Echinacea has not been studied and requires further investigation.

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Table 1.1. Properties of Supercritical Fluids vs. Gases and Liquids[1]

	Gas	SF	Liquid
Density (g/cm^3)	10^{-3}	0.1 – 1	1
Diffusion Coeff. (cm^2/s)	10^{-1}	$10^{-3} - 10^{-4}$	$<10^{-5}$
Viscosity (g/cm s)	10^{-4}	$10^{-3} - 10^{-4}$	10^{-2}

[1] From McNally and Bright (1992).

Table 1.2. Some of the Variables which may Affect SFE Efficiencies[1]

Analyte concentration	Mode of analyte accumulation
Analyte type	Modifier concentrations
Extraction cell agitation (e.g. sonication)	Pressure (density)
Extraction cell dead volume	Restrictor type
Extraction cell dimensions (I.D.:Length)	Sample size
Extraction cell size	Sample matrix (sorbent, co-extractants)
Extraction fluid	Sample particle size
Extraction time	Sample condition (humidity, pH, etc.)
Fluid flow rate	Temperature
Fluid modifiers	Total volume of extraction fluid

[1] From Furton and Rein (1992).

Table 1.3. Critical Conditions for Various Supercritical Solvents[1]

Solvents	Critical Temperature (°C)	Critical Pressure (MPa)
Carbon dioxide	31.1	7.38
Ethane	32.2	4.88
Ethylene	9.3	5.04
Propane	96.7	4.25
Propylene	91.9	4.60
Cyclopropane	124.7	5.49
Isopropanol	235.2	4.76
Chlorotrifluoromethane	28.9	3.87
Benzene	289.0	4.89
Toluene	318.6	4.10
Ethanol	240.8	6.14
Methanol	239.5	8.09
Ammonia	132.5	11.35
Water	374.2	22.12

[1] From Brunner (1994) and McHugh and Krukonis (1994).

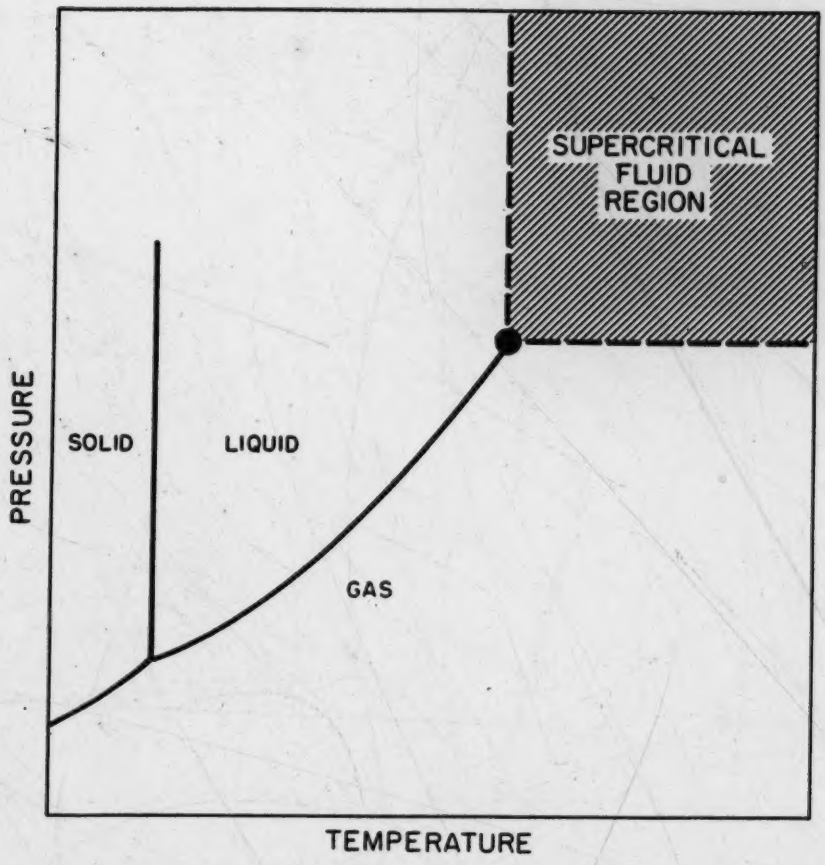

Figure 1.1. Pressure-Temperature Diagram for a Pure Compound

(From McHugh and Krukonis, 1994).

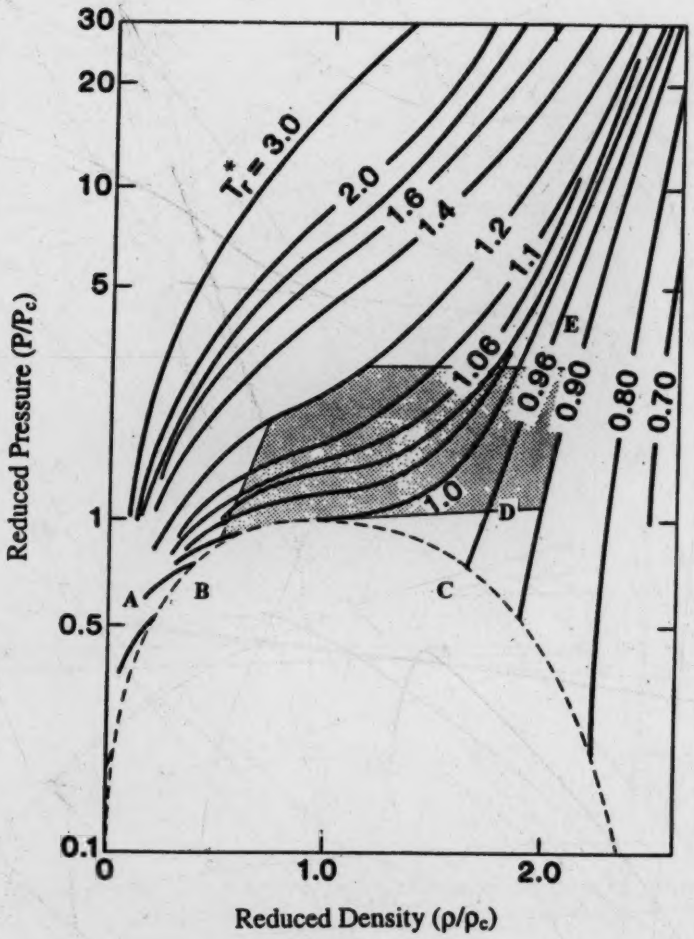

Figure 1.2. Reduced Density of Carbon Dioxide as a Function of Reduced Temperature and Reduced Pressure (* Reduced Temperature, $T_r = T/T_c$)

(From Ely and Baker, 1993).

2. ETHANOL EXTRACTION OF *E. ANGUSTIFOLIA* ROOTS[1]

2.1. INTRODUCTION

Echinacea is native to North America and was used by the Native Americans as remedies for wounds, snakebites, headache and colds (Foster, 1991). Echinacea products have been the best selling herbal supplements in U.S. health food stores in 1996, 1997 and 1998 (Brevoort, 1996, 1998; Richman and Witkowski, 1998). These products are prepared from three species, *Echinacea angustifolia*, *E. purpurea* and *E. pallida*, which have significant healing properties, such as upper respiratory infections (Lindenmuch and Lindenmuth, 2000). However, the effect of extraction conditions on the recovery of Echinacea components is not available in the literature.

Echinacoside (Fig. 2.1) is a caffeic acid derivative, commonly found in Echinacea, especially in the roots. It has shown to have antibiotic activity against streptococci and *Staphylococcus aureus* and was first isolated by Stoll *et al.* (1950). Even though it has not been confirmed that echinacoside has a direct immunostimulating effect, there is evidence that Echinacea powder, which contains echinacoside, acts against viruses (Eilmes, 1976).

Echinacoside is susceptible to hydrolysis during extraction and analysis. Therefore, water is not normally used as an extraction solvent. Phenolic compounds, like echinacoside, are more stable against oxidation in acid solution. Light also contributes to oxidative instability of these compounds. Therefore, acidic preparation conditions and minimal exposure to light would make echinacoside extracts more stable (Baugh and Ignelzi, 2000).

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[1] A version of this chapter is to be submitted to the Journal of Agricultural and Food Chemistry for consideration of publication.

Cynarine (Fig. 2.1) is another unique phenolic compound found in *E. angustifolia* roots and was first reported by Bauer *et al.* (1988). It is a quinic acid derivative and has been suggested as a compound to differentiate *E. angustifolia* from *E. pallida*. However, not much is known about its chemical characteristics or its content in *E. angustifolia*.

Bergeron *et al.* (2000) conducted a study on the recovery of echinacoside and cichoric acid in Echinacea. With 5 min ultrasonic extraction method using ethanol: water (7:3, v/v), the recovery rate obtained was 85% for echinacoside and 89% for cichoric acid from *E. angustifolia* roots. Ethanol was found to cause the least changes whereas water caused the greatest degradation of the compounds in the extraction of phenolic acids (Sauvesty *et al.*, 1992).

Pietta *et al.* (1998) studied the range of caffeic acid derivatives in Echinacea species, including echinacoside and cynarine. They developed micellar electro-kinetic chromatography (MEKC) analysis method for characterizing *E. purpurea*, *E. angustifolia* and *E. pallida* with the lowest detectable amount of 1 μg/mL. A more recent study examined the effect of solvent mixture and its temperature, solvent to feed ratio as well as the particle size on the extraction of alkylamides and cichoric acid of dried *E. purpurea* roots (Stuart and Wills, 2000). Water:ethanol mixture of 40:60 (v/v) resulted in the highest total yield of alkylamides and cichoric acid. Increasing solvent to feed ratio from 2:1 to 8:1 caused an increase for both group of compounds and increasing solvent temperature from 40°C to 60°C yielded more cichoric acid but not alkylamides; reduction of particle size led to an increase in the yield.

E. angustifolia has more alkylamides accumulated in the roots among the three species that are commercially utilized (Bauer and Remiger, 1989; Hobbs, 1989) and

contains a comparable amount of echinacoside to that of *E. pallida* (Bauer *et al.*, 1988). Little is known, however, about the extraction of echinacoside and cynarine from *E. angustifolia* roots. Therefore, the objective of this study was to investigate the effect of the extraction temperature, time and solvent to feed ratio on the extraction of echinacoside and cynarine from *E. angustifolia* roots, using ethanol as a solvent.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Cleaned fresh three-year-old roots of *E. angustifolia* were obtained within 24-48 h of harvest from the Crop Diversification Centre-South, Alberta Agriculture, Food and Rural Development (Brooks, AB). Moisture and O_2-barrier bags (3 MIL, 75% polyethylene and 25% nylon) for vacuum packaging were obtained from Unipack (Edmonton, AB). Anhydrous ethanol, methanol (HPLC grade) and acetic acid (ASC grade) were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2.2. Processing of Echinacea Roots

Echinacea roots were cut into approximately 1-cm long pieces, thoroughly mixed and vacuum packaged. Half of the sample was stored in a –18°C walk-in freezer before being freeze-dried (FFD-42-WS, The Virtis Co. Inc., Gardiner, NY) for three days. The other half was dried for 7 h in a thin-layer air-dryer (Agricultural Value-added

Engineering Centre, Edmonton, AB) at 50°C and air velocity of 1.1 m/s. The moisture content of fresh roots, air-dried and freeze-dried roots was determined in duplicate according to the AOAC official method 930.15 (AOAC, 1999). Dried roots were vacuum packaged in vacuum bags with Multivac (AG500, Sepp Haggenm¨ller KG, Germany) and stored at –18°C until used.

Dried root samples were ground using Osterizer grinder (Model 423 28, Sunbeam Co. Ltd., Canada) for a total grinding time of 40 s but allowing the grinder to cool down after every 10 s. Root samples were referred to as air-dried roots unless specified as freeze-dried roots.

2.2.3. Methanol Extraction of Echinacoside and Cynarine

Methanol extraction was used for analytical purposes to determine the total echinacoside and cynarine content of dried roots. Approximately 1 g of root powder sample was weighed into the 33×80 mm cellulose thimbles (Whatman International Ltd., Maidstone, England), which were then placed in the SOX-TEC soxhlet extraction system (HT2, 1045 Extraction Unit, Tecater, HÖGANÄS, Sweden). The thimbles with root sample were soaked in 70 mL of boiling methanol (64.5°C) and extracted for 1 h, followed by 15 min rinsing. The extracts were quantitatively transferred from the SOX-TEC extraction cups into 100 mL flat bottom flasks and evaporated by a rotary vacuum evaporator at a temperature not higher than 23°C.

2.2.4. Ethanol Extraction of Echinacoside and Cynarine

Ethanol extraction of *E. angustifolia* roots was carried out at 25, 50 and 75°C (water bath temperature) for 30, 60 and 90 min using solvent to feed ratios of 10:1 and 20:1 (v/w). Extraction temperatures levels were selected with respect to room temperature, temperature near boiling point of ethanol (78.5°C) and the temperature in-between. Extraction time was determined according to the methanol extraction method with 30 min shorter or longer. A solvent to feed ratio of 10:1 is commonly applied in industrial production and the ratio 20:1 was used to compare of the ratio effect for the extraction of echinacoside and cynarine. The actual ethanol concentration in the final mixture would vary slightly depending on the moisture content of the feed material.

Extraction experiments were carried out in duplicate at the combinations of conditions shown in Table 2.1 using air-dried roots. During ethanol extraction, it was observed that the actual temperature of the content of flasks was always 1-2°C lower than that of the water bath. Therefore, the extraction temperatures reported were the water bath temperature, unless otherwise stated.

Ethanol extraction was set up similar to the soxhlet extraction, but a 500 mL flat bottom flask sitting in a water bath replaced the extraction cup. The flask had two openings, connected to a condenser and a thermometer. The temperature of water bath was controlled by Thermomix 1420 (B. Braun, Melsungen AG, W. Germany). A magnetic stirrer was used to thoroughly mix the solvent and the sample.

After the extraction, the extract was filtered through Whatman No. 5 filter paper under vacuum and the flask was washed with absolute ethanol before the eluant was

transferred to a 100 mL flat bottom flask. The solvent was removed by a rotary vacuum evaporator and the extract was subjected to the same treatments as those after methanol extraction. Duplicate extraction experiments were also conducted at 75°C for 120 min with a solvent to feed ratio of 10:1 to determine the effect of extended extraction time. Ethanol extraction of freeze-dried roots was also carried out using the best extraction conditions obtained with the air-dried roots.

2.2.5. Sep-Pak Cleanup

Sep-Pak +C_{18} cartridges (Waters Co., Milford, MA) were mounted on a vacuum manifold (Visiprep DL™, Supelco Inc., Bellefonte, PA) and activated by 10 mL methanol, followed by 10 mL Milli-Q water. The extract residue was re-dissolved in 6 mL Milli-Q water, after which 1 mL of methanol was added. The re-dissolved extracts were transferred to a syringe on top of a Sep-Pak cartridge. The flask was washed with 3 mL aqueous methanol (water:methanol, 6:1), which was combined with the previous 7 mL solution. In effect, therefore, the evaporated extract residue was re-constituted in aqueous methanol (6:1) and applied to the activated Sep-Pak. The eluate was collected in a 15 mL test tube. The Sep-Pak cartridge was further eluted with 10 mL of 5:1, 30 mL of 4:1 and 10 mL of 3:1 water:methanol (W:M) solutions, respectively, and collected into designated test tubes. The contents of the first two tubes, with W:M ratios of 6:1 and 5:1 were combined and became the first fraction in HPLC analysis. Collections from W:M ratios of 4:1 and 3:1 were the 2nd and 3rd fractions, respectively.

2.2.6. High Performance Liquid Chromatography (HPLC) Analysis

Three fractions from Sep-Pak fractionation were analysed by HPLC. The HPLC analysis was performed using a UniPoint HPLC computer system for data handling equipped with a 717 Plus auto-sampler (Waters Co., Milford, MA), model 805 pump (Gilson Mandel Scientific, Villiers, Le Bel, France), model 811C dynamic mixer (Gilson Mandel Scientific, Middleton, WI) and JASCO UV-975 intelligent UV/VIS detector (Japan Servo Co., Ltd., Singapore) operated at 330 nm detection wavelength. The system was equipped with a Prodigy C_{18}, 5 µm, 150×4.6 mm, reverse phase column (Phenomenex, Torance, CA) at ambient temperature. The mobile phase consisted of aqueous methanol (W:M = 2.5:1, v/v) with 1% (v/v) of acetic acid at the flow rate of 1 mL/min.

Echinacoside isolated and purified in the lab was used as standard as it is not commercially available. Most of the echinacoside was found in the 2nd fraction, but smaller amounts were also found in the 1st and 3rd fractions and the total amount in all three fractions was reported. Echinacoside standard concentration was monitored by a standard control chart (Fig. 2.2). Cynarine was also isolated later as the standard for quantitation purposes.

2.2.7. Extraction Yield and Recovery

The yield of echinacoside or cynarine was calculated as follows:

$$\text{Yield} = \frac{\text{wt. of echinacoside or cynarine extracted with EtOH}}{\text{wt. of feed sample (dry matter)}} \times 100\%$$

The recovery of these two compounds were defined as follows:

$$\text{Recovery} = \frac{\text{wt. of echinacoside or cynarine extracted with EtOH}}{\text{wt. of echinacoside or cynarine present in the feed sample}} \times 100\%$$

2.2.8. Statistical Analysis

All extraction runs were carried out in duplicate. Analysis of variance of echinacoside and cynarine yields was performed using the General Linear Model procedure of SAS Statistical Software, Version 8 (SAS Institute Inc., 1999). The model variables consisted of extraction temperature, time, solvent to feed ratio and interactions between these main factors. The LSD (Least Significant Difference) test was applied for multiple comparisons of means at $\alpha = 0.05$.

2.3. RESULTS AND DISCUSSION

The moisture content of the fresh roots was 75.31% (w/w) and was reduced to 8.17% and 4.86% by air-drying and freeze-drying, respectively. Drying of roots enabled minimal degradation of echinacoside and cynarine, the two phenolic compounds of interest in Echinacea roots (Bauer, 1998).

The Sep-Pak cleanup procedure allowed all of the echinacoside and cynarine to be eluted into the three fractions, while most non-polar compounds in the extracts were retained in the Sep-Pak cartridge. Ethanol extracts were somewhat slower to elute

through the cartridge than methanol extracts because non-polar compounds were extracted to a greater extent with ethanol.

Figure 2.3 shows typical chromatograms from HPLC analysis of echinacoside and cynarine. The cynarine peak appeared only in the first fraction, while the echinacoside peak was present in the 2nd and 3rd fractions. Echinacoside and cynarine contents of air-dried *E. angustifolia* roots based on methanol extraction were 0.97% (w/w) and 1.61% (w/w) and for freeze-dried roots their contents were 1.30% and 2.07%, respectively (Table 2.2). The results of the best recovery rates of echinacoside and cynarine using ethanol as solvent are shown in Table 2.2.

Analysis of variance results for both echinacoside and cynarine yields are shown in Table 2.3. The statistical comparisons of mean values for echinacoside and cynarine yields of air-dried *E. angustifolia* roots at different ethanol extraction temperatures are presented in Tables 2.4 and 2.5.

2.3.1. Echinacoside Extraction

The echinacoside content (0.97% and 1.30% for air-dried and freeze-dried roots, respectively) determined by methanol extraction was consistent with levels determined by Bauer and Wagner (1991), who claimed there was 0.3-1.3% echinacoside in the roots of *E. angustifolia*. Extraction temperature, time and solvent to feed ratio all had a significant effect ($p \leq 0.05$) on the echinacoside yield while interaction effects were not significant. Extraction time (30, 60 and 90 min) and solvent to feed ratio (10:1 and 20:1)

had different effects on the echinacoside yield at different extraction temperatures (25, 50 and 75°C).

2.3.1.1. Effect of Extraction Temperature

Extraction temperature had a very highly significant effect ($p \leq 0.001$) on echinacoside yield. Yields increased with increasing extraction temperature (25, 50 and 75°C).

At 25°C, the echinacoside yield ranged from 0.17-0.31% while at 50°C its yield was more than doubled, ranging from 0.50 to 0.64% (Table 2.4). Echinacoside yield was further increased at 75°C (0.62-0.82%). Higher temperature resulted in a higher yield of echinacoside and this finding is in agreement with Stuart and Wills (2000). They found that extraction of cichoric acid, another caffeic acid derivative in *E. purpurea*, was enhanced by elevated solvent temperature, from 30% (recovery as percentage of raw material) at 20°C to 45% at 60°C. However, they used ethanol containing 40% (v/v) water as extraction solvent, which may have caused hydrolysis of cichoric acid and affected the yield.

2.3.1.2. Effect of Extraction Time

Echinacoside yields of the extracts obtained from 60 and 90 min extractions were similar, but higher ($p \leq 0.01$) than those from 30 min extraction. However, Table 2.4 revealed that extraction time had different effects on echinacoside yields when ethanol extractions were conducted at different extraction temperatures.

Statistical analysis showed that at 25°C, extraction time did not have a significant effect on the echinacoside yield. The echinacoside yield increased slightly with increasing extraction time. When solvent to feed ratio was 10:1 at 25°C, the echinacoside yield of 60 min extraction was 0.08% greater than that of 30 min extraction.

When extractions were conducted at 50°C and 75°C, echinacoside yields were higher ($p \leq 0.05$) at 60 and 90 min compared to that of 30 min. At 50°C and a solvent to feed ratio of 10:1, extractions conducted for 30 min and 60 min yielded similar echinacoside levels (0.50% and 0.52%). When the extraction time was prolonged to 90 min, echinacoside content was increased from 0.52% to 0.55%, which was significantly different ($p \leq 0.05$) from that of 30 min extraction (0.50%). When solvent to feed ratio was increased to 20:1, similar extraction time effects were observed on the echinacoside yield.

In order to find out the effect of longer extraction time on the echinacoside yield, duplicate experiments were conducted at 75°C with a solvent to feed ratio of 10:1 for 120 min. This increase in extraction time from 90 to 120 min led to a decrease in the echinacoside yield from 0.82% to 0.79%, likely due to heat-degradation of echinacoside.

Stuart and Wills (2000) studied the recovery of cichoric acid from *E. purpurea* using 15 min extraction time in preheated aqueous ethanol solvent at 40, 50 and 60°C. In the present experiment, extraction time of 30 min was too short to optimally extract the echinacoside from the *E. angustifolia* roots. It was possible, therefore, that the cichoric acid extraction conditions used by the others might have been inadequate.

2.3.1.3. Effect of Solvent to Feed Ratio

Generally, a higher solvent to feed ratio of 20:1 gave a better recovery of echinacoside than those from the lower ratio of 10:1. The solvent to feed ratio also had a different influence on the echinacoside yield at different extraction temperatures, as was observed in the effect of the extraction time (Table 2.4).

However, unlike extraction time, solvent to feed ratio had a significant effect ($p \leq 0.05$) on the echinacoside yield only at 50°C, but not at 25°C or 75°C. The higher ratio (20:1) gave a higher recovery of echinacoside due to the availability of more solvent; hence, more solute was extracted. Stuart and Wills (2000) also found that the yield of alkylamides and cichoric acid increased significantly with increasing the solvent to feed ratio from as low as 2:1, to as high as 8:1. They observed that the presence of water in the solvent caused 'swelling' of *E. purpurea* roots and inhibited the remaining solvent to mix freely, thus reducing the solvent mobility. They also observed that when ethanol content was >90% no 'swelling' effect was observed.

Echinacoside recovery was the highest (84.5%) (Table 2.2) when extraction was conducted at 75°C for 90 min, using a solvent to feed ratio of 10:1. The echinacoside yield under this condition was 0.82%. Therefore, it was concluded that optimal extraction conditions for echinacoside in ethanol were 75°C extraction temperature, 90 min extraction time, and 10:1 solvent to feed ratio.

2.3.2. Cynarine Extraction

Similar to the echinacoside yield, extraction temperature, extraction time and solvent to feed ratio of ethanol extraction had a significant effect ($p \leq 0.05$) on the cynarine yield. The relationship between the extraction time and solvent to feed ratio on cynarine yield at different extraction temperatures is illustrated in Table 2.5.

2.3.2.1. Effect of Extraction Temperature

Extraction temperature had a highly significant effect ($p \leq 0.001$) on cynarine yield as was observed with echinacoside extraction. The highest extraction temperature, 75°C, was the best extraction temperature for the extraction of cynarine, as it was for echinacoside.

The cynarine yield at 25°C ranged from 0.24% to 0.43% and at 75°C, ranged from 0.71% to 1.10%. However, there were larger variations when the extractions were conducted at 50°C. When the solvent to feed ratio was 20:1, the cynarine yield was 0.81% after 30 min extraction, and decreased to 0.60% and 0.65% when extractions were conducted for 60 and 90 min, respectively. When solvent to feed ratio was changed to 10:1, the yield of cynarine increased from 0.40% to 0.46%, and 0.51% ($p > 0.05$) as the extraction time was increased from 30 min to 60 and 90 min, respectively.

2.3.2.2. Effect of Extraction Time

The effect of extraction time on cynarine yield was different from that observed for the echinacoside yield. At relatively lower temperature (25°C and 50°C), extraction time had no significant effect ($p>0.05$) on cynarine yield (Table 2.5).

However, when the extraction temperature was increased to 75°C, cynarine yields generally increased with extraction time. At 75°C and both solvent to feed ratios of 10:1 and 20:1, cynarine yield increased significantly ($p\leq0.05$) when extraction time was increased from 60 min to 90 min, but there was no difference between 30 and 60 min extraction. The highest yield of 1.10% was achieved with the solvent to feed ratio of 20:1 at 75°C and 90 min extraction. This may imply that the extraction equilibrium between cynarine and ethanol was not reached until after 60 min, resulting in more being solubilized.

2.3.2.3. Effect of Solvent to Feed Ratio

Solvent to feed ratio had a greater impact ($p\leq0.001$) on the recovery of cynarine than on echinacoside (Table 2.3). Solvent to feed ratio of 20:1 yielded significantly higher ($p\leq0.05$) cynarine compared with those from the ratio of 10:1 when extractions conducted at 25°C and 50°C from air-dried roots. The highest cynarine recovery rate was 68.3% (Table 2.2) and it was obtained at the highest extraction temperature (75°C) with solvent to feed ratio of 20:1 and extraction time of 90 min.

Variation was observed in the cynarine yields, which is not surprising since the extraction and analytical procedures were designed to quantify echinacoside. Cynarine yields were calculated based on the data from echinacoside analysis, which included

cynarine peaks, calibrated with the isolated cynarine standard. Furthermore, methanol extraction was the best extraction method for the quantitation of echinacoside which was established two years ago in our lab. Therefore, cynarine quantitation was not optimised in this analysis.

2.3.3. Freeze-dried *E. angustifolia* Roots

Ethanol extraction was also carried out using the freeze-dried *E. angustifolia* roots under the optimal ethanol extraction conditions established for the air-dried roots (at 75°C for 90 min with feed to solvent ratio of 20:1). With ethanol extraction, only 51.5% of echinacoside was recovered from freeze-dried roots (Table 2.2). The cynarine yield obtained was 0.84%, compared with 2.07% from the methanol extraction, resulting in a recover of 40.6%.

Comparing the two dried samples, it was observed that the freeze-dried root powder, with the moisture content of 4.86%, was less dense than the air-dried roots. It was also observed that some of the freeze-dried root powder was floating at the surface of the solvent during the extraction process and tended to stick to the flask wall, resulting in the low extraction efficiency. The solvent was not well mixed with the freeze-dried root powder and failed to penetrate the sample matrix to dissolve the solutes. But Stuart and Wills (2000) reported that a smaller particle size resulted in higher recovery of alkylamides and cichoric acid from the *E. purpurea* roots.

2.4. CONCLUSION

Higher extraction temperature and solvent to feed ratio with reasonably long extraction time will lead to better extraction yield of echinacoside and cynarine in *E. angustifolia* roots. It is concluded that the best extraction conditions in this study are 75°C for 90 min using solvent to feed ratio of 10:1 for echinacoside and 20:1 for cynarine in the roots while the ratio 20:1 yielded the highest combination of these two compounds.

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Table 2.1. Combination of Conditions Used in Ethanol Extraction of Echinacoside and Cynarine

Extraction Time	Water Bath Temperature					
	25° C		50° C		75° C	
	Solvent to Feed Ratio (v/w)					
30 min	10:1	20:1	10:1	20:1	10:1	20:1
60 min	10:1	20:1	10:1	20:1	10:1	20:1
90 min	10:1	20:1	10:1	20:1	10:1	20:1

Table 2.2. Best Recovery Rates of Echinacoside and Cynarine Using Ethanol as Solvent

Echinacea Roots	Echinacoside Yield			Cynarine Yield		
	Highest Yield[1] (% extracted/ g dry roots)	Content (% / g dry roots)	Recovery	Highest Yield[2] (% extracted/ g dry roots)	Content (% / g dry roots)	Recovery
Air-dried	0.82	0.97±0.04	0.85	1.10	1.61±0.05	0.68
Freeze-dried	0.67	1.30±0.00	0.52	0.84	2.07±0.08	0.41

[1] Ethanol extraction conducted at 75° C for 90 min using solvent to feed ratio of 10:1.

[2] Ethanol extraction conducted at 75° C for 90 min using solvent to feed ratio of 20:1.

Table 2.3. Analysis of Variances of Factors Affecting Echinacoside and Cynarine Yield at Different Ethanol Extraction Conditions

Extraction Factors	Echinacoside		Cynarine	
	Mean Square	Significance Level	Mean Square	Significance Level
Temperature	0.733	***	0.866	***
Time	0.032	**	0.027	*
Ratio	0.021	*	0.230	***
Temperature · Time	0.005	NS	0.020	NS
Temperature · Ratio	0.002	NS	0.0112	NS
Time · Ratio	0.0004	NS	0.0126	NS
Temperature · Time · Ratio	0.0006	NS	0.0088	NS

* Results significant at $p \leq 0.05$ level.
** Results significant at $p \leq 0.01$ level.
*** Results significant at $p \leq 0.001$ level.
NS Results not significant at $p > 0.05$ level.

Table 2.4. Yield of Echinacoside* from Air-dried *E. angustifolia* Roots at Different Ethanol Extraction Conditions**

Extraction Temperature	Solvent to Feed Ratio: 10:1			Solvent to Feed Ratio: 20:1		
	Extraction Time			Extraction Time		
	30 min	60 min	90 min	30 min	60 min	90 min
25°C	0.17^{a}	0.25^{a}	0.25^{a}	0.25^{a}	0.27^{a}	0.31^{a}
50°C	0.50^{a}	0.52^{ab}	0.55^{b}	0.57^{a}	0.60^{ab}	0.64^{b}
75°C	0.62^{a}	0.75^{b}	0.82^{b}	0.65^{a}	0.78^{b}	0.81^{b}

$^{a-b}$ Means followed by the same letter in each row are not significantly different at $p > 0.05$ level.

* Yield of echinacoside was expressed in terms of % (w/w, on dried roots).

** Analysis was conducted with respect to different extraction temperatures.

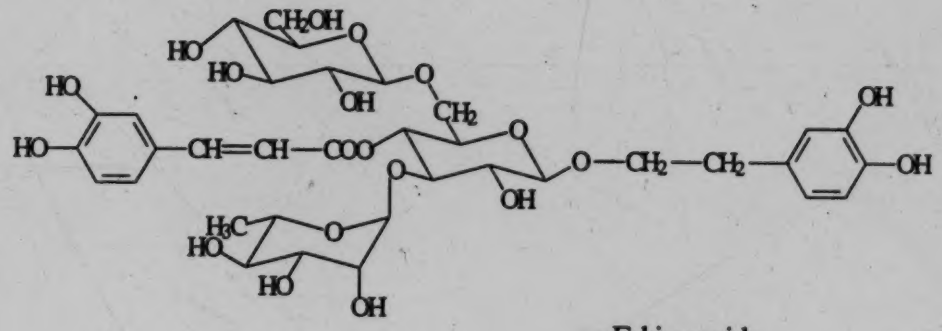
Table 2.5. Yield of Cynarine* from Air-dried *E. angustifolia* Roots at Different Ethanol Extraction Conditions**

Extraction Temperature	Solvent to Feed Ratio: 10:1			Solvent to Feed Ratio: 20:1		
	Extraction Time			Extraction Time		
	30 min	60 min	90 min	30 min	60 min	90 min
25°C	0.28[a]	0.24[a]	0.31[a]	0.43[a]	0.40[a]	0.41[a]
50°C	0.40[a]	0.46[a]	0.51[a]	0.81[a]	0.60[a]	0.65[a]
75°C	0.71[a]	0.83[a]	0.93[a]	0.85[a]	0.86[a]	1.10[a]

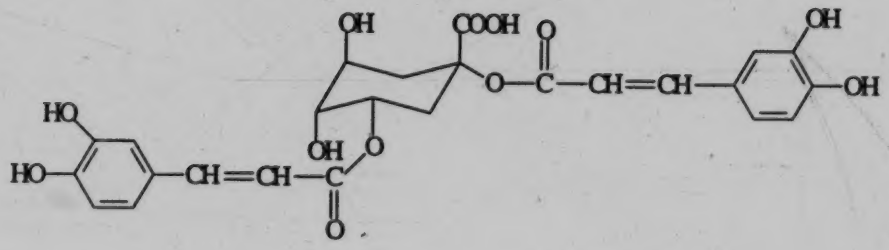
[a-b] Means followed by the same letter in each row are not significantly different at $p>0.05$ level.

* Yield of cynarine was expressed in terms of % (w/w, on dried roots) .

** Analysis was conducted with respect to different extraction temperatures.



Echinacoside



Cynarine

Figure 2.1. Chemical Structures of Echinacoside and Cynarine

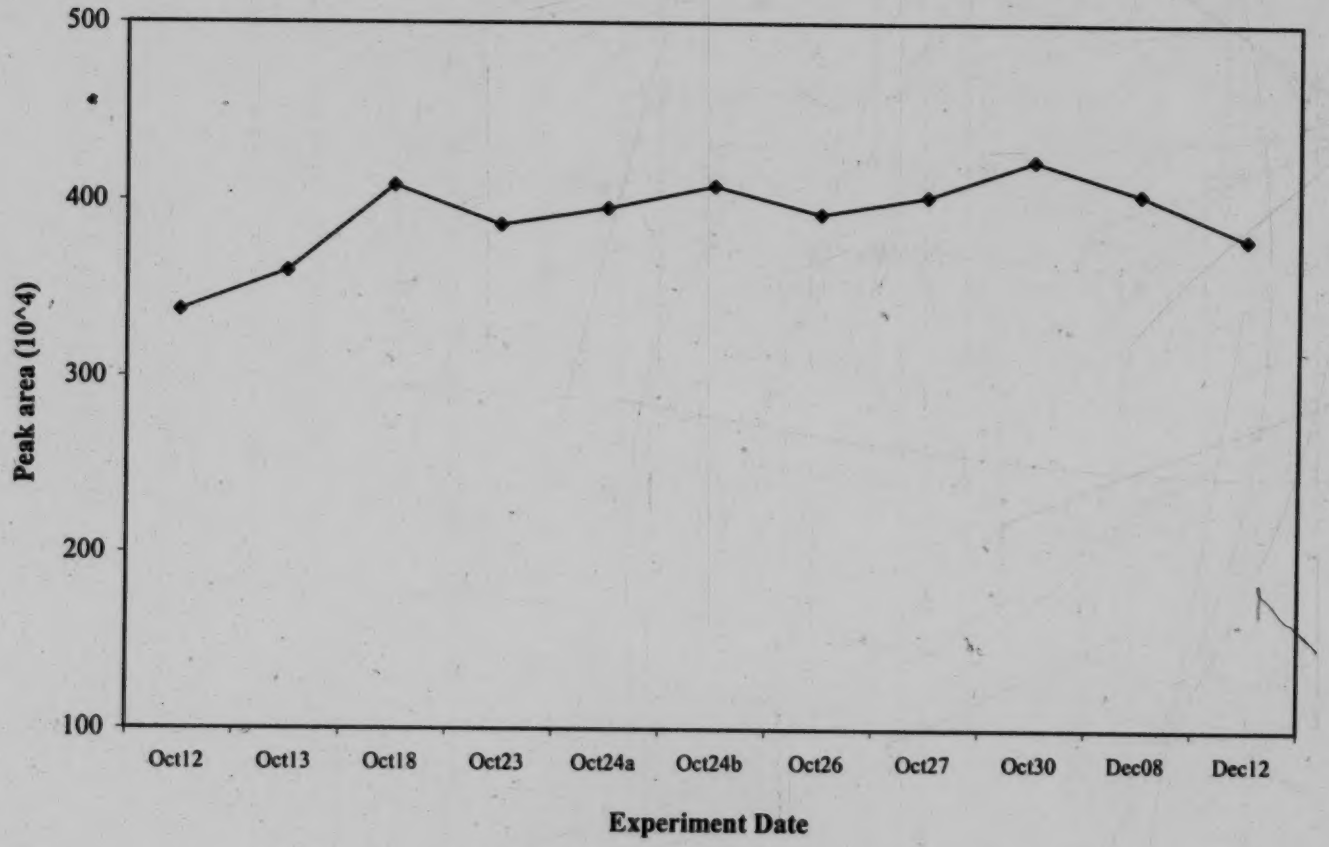
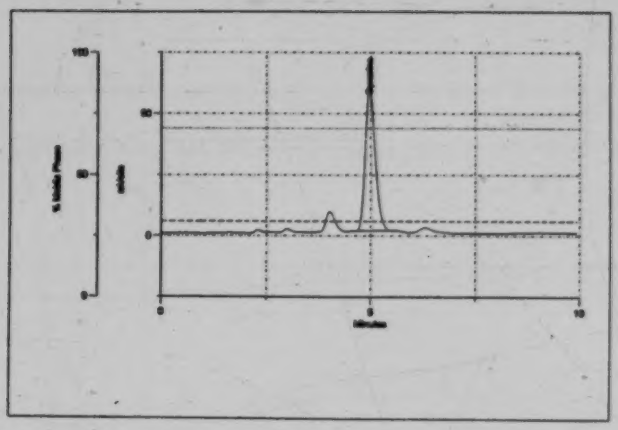
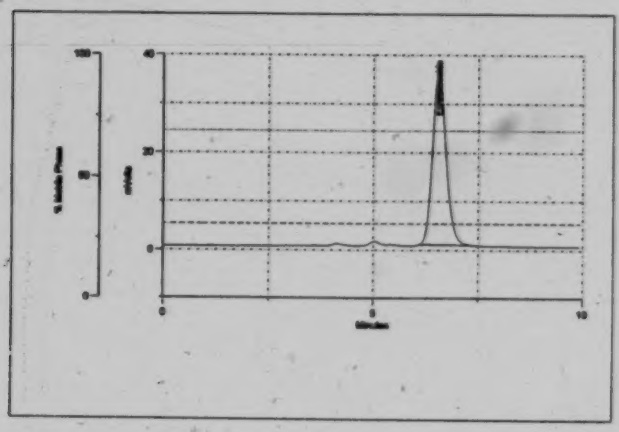


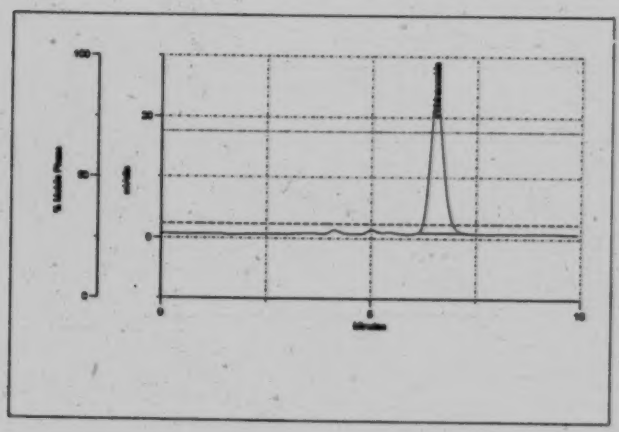
Figure 2.2. Echinacoside Standard Control Chart



1st Fraction



2nd Fraction



3rd Fraction

Figure 2.3. Typical HPLC Chromatograms of Echinacoside and Cynarine Analysis

3. SUPERCRITICAL FLUID EXTRACTION OF *E. ANGUSTIFOLIA* ROOTS[1]

3.1. INTRODUCTION

Herbal therapies have received increasing attention both in the North America and Europe. Although there is not enough scientific evidence to show the efficacy, their use as alternative medicines has been growing steadily. Echinacea is one of the most popular herbal plants which has been extensively studied in recent years. There are more than 800 Echinacea products on the market in Germany alone (Bauer, 1998). Cosmetic products containing Echinacea extracts such as lip balms, shampoos and toothpaste are also available (Leung and Foster, 1996).

Echinacea angustifolia is one of the three species of Echinacea available commercially and has a high market value (Li, 1998; Davies, 1999). It has been used by American Indians for toothaches, headaches, snakebites and other poisonous conditions, even for cold and cancers (Foster, 1991).

Compounds that have been isolated from Echinacea include polysaccharides, caffeic acid derivatives and lipophilic components (Hobbs, 1989; Bauer and Wagner, 1991; Bone, 1997). Alkylamides, a group of lipophilic compounds together with polar fractions contribute to the immunostimulatory activity of Echinacea (Bauer and Wagner, 1991). Several studies have reported the analysis of alkylamides in Echinacea (Bauer *et al.*, 1988a; Bauer and Remiger, 1989; He *et al.*, 1998; Lienert *et al.*, 1998; Pietta *et al.*,

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[1] A version of this chapter is to be submitted to the Journal of Agricultural and Food Chemistry for consideration of publication.

1998). However, more research is needed to find an effective method for the extraction of these compounds from the herb.

Alkylamides have been extracted by soxhlet extraction or maceration using organic solvents, such as methanol (Bauer *et al.*, 1988a; Rogers *et al.*, 1998), chloroform (Bauer and Remiger, 1989; He *et al.*, 1998), hexane (Bauer *et al.*, 1988b, 1989), ethanol or aqueous ethanol (Bergeron *et al.*, 2000; Stuart and Wills, 2000), and aqueous methanol (Bergeron *et al.*, 2000). Most of these studies have been carried out using ground dried roots, either air-dried or freeze-dried. Extraction of fresh roots has not been reported.

Supercritical fluid extraction (SFE) of alkylamides from Echinacea roots was explored by Lienert *et al.* (1998) on an analytical scale. They applied 15.05 MPa and 60°C for 30 min, followed by 30 min of dynamic extraction at a flow rate of 1 mL/min. There was no significant difference between SFE, maceration and soxhlet extraction methods in their study in terms of the composition of the extracts.

SFE is a promising technology in food and pharmaceutical industries. More recently, its applications are expanding, especially in the 'natural' products and nutraceutical areas. Carbon dioxide is the most commonly used supercritical solvent for its moderate critical point (P_c = 7.38 MPa, T_c = 31.1°C), and its non-toxic and environmentally friendly characteristics. Its density varies with pressure and temperature, and preferentially dissolves non-polar or slightly polar components. Supercritical CO_2 has been successfully used commercially in decaffeinating coffee and tea and in the extraction of hops, flavours and other natural materials (Williams, 1981; King and Bott, 1993; Mermelstein, 1999). Despite the rapid developments in the applications of SFE, the extraction of alkylamides from Echinacea has not been reported.

Therefore, the objective of this study was to examine the effect of SFE conditions on the extraction of alkylamides from fresh and dried roots of *E. angustifolia*.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Fresh three-year-old roots of *E. angustifolia* were obtained within 24-48 h of harvest from the Crop Diversification Centre South, Alberta Agriculture, Food and Rural Development, Brooks, AB. Dichloromethane was used to dissolve SFE extracts for further analysis and was purchased from BDH Inc. (Toronto, ON). Hexadecane as an internal standard (99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Carbon dioxide (bone dry) used for the extraction, and UHP helium (ultra high purity, 99.9999%) used as carrier gas in gas chromatograph – mass spectrometer (GC-MS) were obtained from Praxair Canada Inc. (Mississauga, ON). Cerulenin used as external standard (95%) was purchased from Sigma Chemical Co. (St. Louis, MO).

3.2.2. Echinacea Roots Processing

Echinacea roots were cleaned, packaged, stored and dried according to the procedures described previously in Section 2.2.2. Moisture content of fresh and dried roots was determined according to the AOAC official method 930.15 (AOAC, 1999).

Particle size distribution of ground air-dried and freeze-dried roots was determined with a Portable Sieve Shaker (The W.S. Tyler Co. of Canada Ltd., St. Catharines, ON), using sieves of 16, 20, 40, 80 and 100 mesh (Canadian Standard).

3.2.3. Supercritical Fluid Extraction

Supercritical fluid extractions of Echinacea roots were performed using a laboratory scale system (Newport Scientific, Inc., Jessup, MD) as shown in Figure 3.1. The Echinacea sample (approximately 25 g of fresh or 6 g of dried roots) was placed in a sample basket (25 cm x 27 mm I.D.) where glasswool was inserted at both ends to hold the sample. Fresh roots were used as 1-cm long pieces whereas dried roots were ground before use. Dried roots were ground using Osterizer 8 grinder (Sunbeam Co. Ltd., Canada) for a total grinding time of 37 s, allowing the metal stirrer to cool down after every 10 s. The sample basket was then placed into the original extraction chamber. Extraction temperature was monitored within ±2°C of the desired temperature, using a thermocouple placed at the top portion of the extraction cell, a temperature controller and the heater around the extraction cell. A backpressure regulator was used to maintain the desired pressure.

The extractions were performed for 4 h in duplicate at two pressures (34 and 55 MPa) and two temperatures (45 and 60°C). The CO_2 flow rate was measured at ambient conditions and maintained at an average level of 1.67 L/min. A blank run was conducted at 34 MPa and 45°C to determine if there was any contamination of the extracts from the system. There were additional peaks obtained in subsequent chromatographic analysis

which were determined to be due to the contaminants and were excluded in the normalised calculation of the total extracts.

Alkylamide extracts were collected in two side-armed test tubes connected in series held in a cold bath at -10°C. Collection tubes were allowed to equilibrate at room temperature and were weighed 20 min after extraction was completed.

At the extraction conditions of 34 MPa and 60°C, unground air-dried roots were extracted by supercritical CO_2 alone. Duplicate extractions of ground air-dried roots were conducted under the same extraction conditions with the addition of ethanol, introduced prior to solvent entry into the extraction cell, at a rate of 0.21 mL/min, which corresponded to 5% (w/w) of ethanol in CO_2. Ethanol was injected into the flow of CO_2 using a piston pump (Gilson 305; Gilson, Inc., Middleton, WI), which was equipped with a manometric module.

Residues from duplicate runs at the best SFE extraction conditions of air-dried roots were mixed and extracted at the optimal ethanol extraction conditions determined previously (Chapter 2, 75°C for 90 min with solvent to feed ratio of 20:1) to determine the yield of echinacoside and cynarine. Similar ethanol extraction was also carried out using freeze-dried root residues.

3.2.4. Alkylamide Analysis of Extracts

Alkylamides were identified by the comparison of their mass spectra patterns with those previously reported by Bauer *et al.* (1988b, 1989). Identification was done with the assistance of Varian Vista 6000 gas chromatograph (Varian Associate Inc., Walnutcreek,

CA), equipped with a DB 5 column (30 m x 0.24 mm I.D. and 0.25 μm film, J & W Scientific, Folsom, CA) and coupled with a 7070E VG Analytical Mass Spectrometer (V.G. Micromass Ltd., Manchester, UK). Although an exact point-to-point replication of the mass spectra was not obtained, the reported alkylamides were identified to the best possible confidence. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Analytical Systems Inc., Billerica, MA) was also used for confirmation of peak identifications of alkylamides.

Since pure standards of alkylamides of interest in this study were not available commercially, cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide, $C_{12}H_{17}NO_3$, MW = 223.3) was used as an external standard to determine the apparent quantities of the alkylamides detected by GC-MS. In addition, hexadecane was used as an internal standard for quantitation purposes. Hexadecane (100 μL) was dissolved in 5 mL dichloromethane (IS solution) and kept in a sealed glass vial and used for four successive working days. An aliquot of IS solution (100 μL) was added to the extract dissolved in 10 mL CH_2Cl_2 and stored at 4°C until the analysis next day.

A solution containing 0.333 mg of standard cerulenin and 0.315 mg of internal standard hexadecane in 10 mL CH_2Cl_2 was prepared. The solution (5 μL) was injected to GC-MS and retention times of 17 and 34 min were obtained for hexadecane and cerulenin, respectively. The area ratio of cerulenin with respect to hexadecane was 36:100. The relative response factor based on weight is then calculated as IS:STD = 1:2.94 (Annino and Villabobos, 1992).

Analysis of extracts was carried out using a Hewlett Packard (HP) 5890 Series II gas chromatograph, equipped with a DB 5MS column (30 m X 0.25 mm I.D. and

0.25 µm film, J & W Scientific, Folsom, CA) and a HP 5971A mass selective detector (Palo Alto, CA). Helium at 0.34 MPa was used as the carrier gas. Oven temperature was programmed using HP G1034C software for the MS ChemStation (DOS series). The oven was held at 55°C for 3 min, and the temperature was then increased at a rate of 4.5°C/min to 230°C and held for 5 min. The ionisation voltage was 1400 eV and the mass spectrometry was used in the scan mode (1.2 scan/sec). Injection volume was 5 or 1 µL depending on the concentration of the extract solution. HP G1034C software was used to integrate the peak areas of the chromatogram.

For the quantitation of alkylamides in the extracts, 100 µL of the IS solution was added to the extracts dissolved in dichloromethane. The area count of each peak of the chromatograms was normalized to the total internal standard present in each extract solution to obtain relative quantities. The normalized quantities of the components were then obtained by dividing the relative quantities with the relative response factor based on the external standard cerulenin. Thus, the normalized quantities of identified alkylamides in each extract were reported on a dry root weight basis of the original sample.

3.2.5. Statistical Analysis

All extraction runs and analysis of each extract were carried out in duplicate. Analysis of variances of results was performed using the General Linear Model (GLM) procedure of SAS Statistical Software, Version 8 (SAS Institute Inc., 1999). The model consisted of the main effects of pressure, temperature and their interaction. Multiple

comparison of means was carried out using the LSD (Least Significant Difference) test at $\alpha = 0.05$.

3.3. RESULTS AND DISCUSSION

Fresh *E. angustifolia* roots contained 75.31% (w/w) moisture. The moisture content of air- and freeze-dried roots was 8.17% and 4.86%, respectively. Particle size analysis of air-dried and freeze-dried roots is presented in Table 3.1. Approximately 78.82% (w/w) of freeze-dried root particles were smaller than 425 µm, whereas the same fraction made up only 51.08% of the air-dried roots. More particles of air-dried roots (25.86%) were distributed in the size range of 425 to 850 µm than those of freeze-dried roots (18.25%) and particles bigger than 850 µm were found to a larger extent (23.06%) in air-dried roots than in freeze-dried roots (2.93%).

Figure 3.2 shows a typical gas chromatogram of an Echinacea root extract obtained from SFE. Among the over 20 peaks obtained, eight major alkylamides were identified (Fig. 3.3). They were numbered according to the sequence shown in the chromatogram as compound **a** to **g**. Compound **d** is actually a mixture of tetraene alkylamides 14 and 15 reported by Bauer *et al.* (1989) since they were not fully separated. All of these compounds were present in all the extracts obtained under different conditions, except that compound **g** was missing in the extract obtained at 55 MPa and 60°C. The retention times of compounds **a**, **b**, **c**, **d**, **e**, **f** and **g** were 36-37, 40-41, 44-45, 46-49, 50-51, 52-53 and 53-54 min, respectively.

A blank extraction run was carried out without any root sample loaded in the extraction cell and contaminants were found in the extract. GC-MS of dichloromethane alone also resulted in some noise peaks around retention time of 54 min and later. Therefore, GC peaks at retention times of 31 min and ≥54 min, belonged to the contaminants and the solvent introduced during the process of extraction and washing of plastic tubing with organic solvents. These peaks were excluded in the total extract calculations. Peak at 17 min is hexadecane used as internal standard (IS).

Means of duplicate run results of supercritical fluid extraction at each condition were reported and there was no significant difference ($p>0.05$) between the duplicates. The yield of individual compounds and total extracts are reported in terms of mg/g dry root basis to facilitate better comparison among samples. Changing pressure and temperature, as well as the condition and form of samples affected the alkylamide yield. A higher recovery could have been achieved if the extractions were continued longer. There were also some losses during the depressurisation step.

3.3.1. Fresh *E. angustifolia* Roots

Yield of individual alkylamides from fresh roots ranged from 0.03 to 2.13 mg/g dry root. Alkylamides, together with other extracted components were gum-like droplets on the collection tube wall. Water was co-extracted and captured in the collection tube as well, but its amount was not quantified. It also appeared as droplets on the tube wall, which were washed with dichloromethane. This phenomenon was observed by others during SFE of plant materials (Snyder *et al.*, 1984; Dunford and Temelli, 1997).

Analysis of variance showed that both temperature and pressure had a significant effect ($p \leq 0.05$) on the yield of identified alkylamides as a whole yet had slightly different effects on individual components.

3.3.1.1. Effect of Temperature

Figure 3.4 presents the effects of temperature and pressure on the normalized amounts of alkylamides in the supercritical CO_2 extracts of fresh *E. angustifolia* roots. At 34 MPa, when temperature was increased from 45°C to 60°C, the yield of all alkylamides was at least doubled, with compound **c** having the greatest increase (237%). The same temperature increase at 55 MPa caused the yield of compound **g** to decrease by 25% whereas the rest showed increases ranging from 75% to 188%. Compound **b** had the smallest increase while compounds **c, e** and mixture **d** almost tripled. Eight identified alkylamides had different responses to temperature change at 34 MPa and 55 MPa.

Even though the overall yield was lower at higher pressure, individual yields of compounds **a, d** and **e** showed a greater percentage (145%, 176%, 188%) increase at 55 MPa than those at 34 MPa (115%, 151%, 169%) when temperature was raised from 45°C to 60°C. Conversely, yield of compound **g** was reduced at 55 MPa and 60°C compared to that obtained at 55 MPa and 45°C. This may be due to experimental error since its quantity was very low. Yields of all compounds except for compound **g** at 60°C were significantly higher ($p \leq 0.05$) than those at 45°C at both pressure levels.

Yield of alkylamides does not seem to be affected by their physical states since compounds **a, c** and **g** are colorless oil at room temperature whereas the rest are colorless crystals (Bauer *et al.*, 1989). This is due to increased vapor pressure of alkylamides so

that it is easier to be solubilized by supercritical CO_2. Furthermore, increasing temperature caused increased diffusion coefficient of supercritical CO_2 so that it was easier to penetrate into the roots and to dissolve more alkylamides and other components present in the *E. angustifolia* roots.

A similar temperature effect was obtained for the total extract. Including unidentified compounds, approximately 2.5 times more extract was obtained at 60°C at a pressure of 34 MPa (7.71 mg/g dry root) than at 45°C (2.91 mg/g dry root). A similar increase was found at 55 MPa when temperature was increased from 45°C to 60°C (1.9 and 4.67 mg/g dry root). Total extract yield increased with temperature at both 34 MPa and 55 MPa except that the increase at 34 MPa was slightly higher than that at 55 MPa.

3.3.1.2. Effect of Pressure

As mentioned above, yield of alkylamides from fresh roots at 34 MPa was higher than that at 55 MPa. At 45°C, a decrease in pressure from 55 MPa to 34 MPa led to an increase in the yield of eight alkylamides ranging from 21% to as high as 128%. The percentage increase in yield was doubled when extractions were carried out at 60°C, where the increase ranged from 41% to 170% with a drop in pressure from 55 MPa to 34 MPa. Therefore, the pressure effect on the extraction of alkylamides from fresh roots was enhanced at higher extraction temperatures. Increasing pressure led to an increase in supercritical CO_2 density, which should correspond to a higher yield of alkylamides. However, there is a simultaneous increase in the solubility of water in supercritical CO_2, which also acts as a barrier to the extraction of alkylamides from the cell structure of the roots (Dunford *et al.*, 1997).

which also acts as a barrier to the extraction of alkylamides from the cell structure of the roots (Dunford *et al.*, 1997).

Further examination of individual compounds revealed that compounds **a, d** and **e** had lower increases in yield at 60°C, 41%, 54% and 59% compared to 61%, 70% and 71% at 45°C, respectively, with a drop in pressure. Other than the yield of alkylamides **a**, **b** and **g**, the yields of the remaining compounds at 55 MPa were significantly lower ($p \leq 0.05$) than those at 34 MPa. The temperature*pressure interaction did not have a significant effect ($p > 0.05$) on the yield of individual compounds and total extracts.

At 34 MPa and 60°C, a higher extract yield was obtained from fresh roots. This was true for total extracts as well as the eight major compounds identified. Total extracts were reduced by at least 53%, from 7.71 to 4.67 mg/g dry root at 60°C and 2.91 to 1.90 mg/g dry root at 45°C, when the pressure was increased from 34 MPa to 55 MPa. Fresh *E. angustifolia* roots resulted in enhanced yield at lower pressure and higher temperature (i.e., 34 MPa and 60°C). Quantitation of the co-extracted water from fresh roots at different SFE conditions needs to be studied further.

3.3.2. Dried *E. angustifolia* Roots

It was observed that extracts from fresh roots dissolved in dichloromethane were light yellow in colour compared to bright yellow for those of dried Echinacea roots. This indicates that some colour pigments are extracted easier from dried samples. Unlike alkylamide yields from fresh roots, yields from dried roots were at a much higher level, ranging from 0.52 to 12.28 mg/g dry root. Figure 3.5 presents the temperature and

pressure effects on the normalized yield of alkylamides in supercritical CO_2 extracts of air-dried *E. angustifolia* roots. The GC peaks were less sharp compared with those from fresh roots and retention times of the eight identified alkylamides were slightly delayed. Analysis of variance proved that temperature and pressure had significant effects ($p \leq 0.05$) on dried root alkylamide yields.

3.3.2.1. Effect of Temperature

Temperature had a similar effect on the yield of alkylamides and total extract from dried roots as those on fresh roots. At 34 MPa, the yield of compounds **b**, **f** and **g** had a slight change whereas the remaining five compounds showed 18-24% increase when temperature was changed from 45°C to 60°C. Increasing pressure to 55 MPa, there was a yield increase in every compound ranging from 19% to 62% where compound **f** showed the greatest increase. Compounds **c** and **d** had the same yield increase at both pressure conditions due to temperature change. The yield of only alkylamide **e** was significantly higher ($p \leq 0.05$) at 60°C compared to 45°C.

Stuart and Wills (2000) studied the solvent temperature effect during the aqueous ethanol extraction of alkylamides from dried *E. purpurea*. They found the optimal extraction of alkylamides was at 20°C with 60% recovery from the raw material compared with 35% at 60°C, which indicated a decrease of alkylamides recovery with elevated extraction temperature. Study of the extraction residue further indicated that there was considerable degradation of alkylamides during the extraction process (Stuart and Wills, 2000).

3.3.2.2. Effect of Pressure

Pressure had a different effect on the yield of alkylamides and total extracts from dried roots compared to the results of fresh roots. At 45°C, increasing extraction pressure from 34 MPa to 55 MPa resulted in a yield increase (24-56%) of all eight compounds with compound **c** having the highest percentage increase. At 60°C, 30-112% of yield increase was achieved when pressure was increased from 34 MPa to 55 MPa. Compounds **c** and **d** had almost the same increase at the two temperatures due to pressure increase. Yields of three compounds **a**, **e** and **g**, were significantly increased ($p \leq 0.05$) by pressure.

Figure 3.6 shows a comparison of the yield of total extract obtained from fresh and air-dried roots from supercritical CO_2 extraction at different conditions of this study. At 55 MPa and 45°C, which was the most unfavorable extraction condition for fresh roots, there was over a 10 fold increase of total extract from air-dried roots. These results indicate that it is much easier to extract alkylamides and other components from dried roots using supercritical CO_2.

3.3.2.3. SFE of Air-dried Roots Using Ethanol as an Entrainer

In order to enhance the solvent power of CO_2 and increase its polarity, ethanol was injected and mixed into the flow of CO_2 before it entered the extraction cell. Ethanol, together with water, is the best 'natural' entrainer for food-grade products (Moyler, 1993). Water has been successfully used in the coffee decaffeination process to optimize the caffeine removal by steaming the green beans to a moisture content of 40-45% from 10-12% (Lack and Seidlitz, 1993).

Supercritical CO_2 extraction using ethanol (5%, w/w) as an entrainer was conducted at 34 MPa and 60°C with air-dried roots. The yield of alkylamides ranged from 0.54 to 8.43 mg/g dry root. Except for compounds **b, c** and **e,** the yields of other alkylamides were reduced when compared with the yields obtained from the same roots without ethanol addition. Overall, there was no significant difference ($p>0.05$) on the yield of alkylamides obtained with or without ethanol addition.

3.3.2.4. Comparison of Ground and Unground Air-dried Roots

The alkylamide yields obtained from unground air-dried roots (Fig. 3.7) were surprisingly low, even less than those from fresh roots at 34 MPa and 45°C. At 34 MPa and 60°C, the yield of alkylamides from unground air-dried roots were between 0.09 to 0.78 mg/g dry root and total extract yield was 2.09 mg/g dry root. Compared to yields from ground air-dried roots, those from unground roots were significantly lower ($p\leq0.05$) for every individual compound.

Even though water acted as a barrier for supercritical CO_2 to penetrate into the roots and dissolve the solute in fresh roots, unground air-dried roots had a more critical structure. After drying, the woody surface of the roots drew back and the cell walls shrunk so that the roots became crumpled and the vessels in roots were also blocked. However, grinding can greatly increase the surface area of the roots in contact with the supercritical CO_2 and lead to an increase in the alkylamide yields. Those factors may contribute to the low yield of alkylamides from unground air-dried roots.

3.3.2.5. Comparison of Fresh and Dried Roots

It is apparent from the above discussion that the best SFE conditions depend on the sample moisture level. i.e., 34 MPa and 60°C was best for fresh roots and 55 MPa and 60°C was best for dried roots. SFE of freeze-dried roots was conducted at the best extraction conditions of air-dried roots since its moisture content (4.86%) was comparable to that of air-dried samples (8.17%). Figure 3.8 gives a comparison of the yields of eight identified alkylamides from fresh roots, air-dried roots and freeze-dried roots at their best supercritical extraction conditions.

It was clear that extracts of dried roots contained more alkylamides than those of fresh roots. Freeze-dried root extracts seemed to have slightly more alkylamides than those of air-dried roots for most identified compounds. Analysis of variance of the total extract for the sample effect showed that fresh roots were highly significantly ($p \leq 0.01$) different from both dried roots, while air-dried and freeze-dried roots were similar. This is consistent with the Atlantic mackerel study done by Dunford *et al.* (1997) where they found that the oil extract yields from mackerel containing 10.2% and 3.8% moisture were similar (2.5-2.7g). Snyder *et al.* (1984) also had a similar finding on supercritical CO_2 extraction of soybeans at moisture levels of 3% and 12%.

When fresh root extracts were compared with those of air-dried roots at their best respective extraction conditions, there was 166-658% increase in the yields of eight individual alkylamides and 350% increase in the yield of total extract. Compounds **c, d** and **g** increased at least four times indicating that it is much easier to extract alkylamides from dry roots rather than from fresh roots.

Water is immiscible with supercritical CO_2 but still dissolved to a small extent (Lehotay, 1997). In the case of fresh roots, water was acting as an entrainer in the extraction process. Unfortunately, even though water kept the roots in shape, it dissolved in supercritical CO_2 so that it reduced the solubility of alkylamides in the roots. Furthermore, fresh roots were in 1-cm chunks with insufficient surface area for the solvent to penetrate into the matrix to facilitate efficient extraction. On the other hand, powdered dried roots were the ideal matrix for SFE (Smith, 1999). Grinding had destroyed the physical state of the roots into fine powder with good permeability and enabled interactions between solvent and roots over a large surface area.

Bauer *et al.* (1989b) found that compound mixture **d** is the major alkylamides in *E. angustifolia* at a level of about 0.009-0.151% (Bauer and Remiger, 1989). The yield of mixture **d** using SFE was within the range of 0.50 to 14.34 mg/g dry root. In both sample forms, compound mixture **d** remained to be the alkylamides of the highest yield. The top five yield of compounds **d**, **a**, **e**, **c** and **b** corresponded to and was consistent with the top five compounds extracted by hexane, **14** and **15**, **2**, **10**, **1** and **3** by Bauer *et al.* (1989), where alkylamide **e** was more efficiently extracted by SFE compared to *n*-hexane.

3.3.3. Ethanol Extraction of SFE Residues

Ethanol extraction of SFE residues was conducted at 75°C for 90 min using solvent to feed ratio of 20:1 to examine the retention of echinacoside and cynarine after SFE. As expected, the re-dissolved solutions from the evaporated ethanol extracts from SFE residues were much clearer and eluted Sep-Pak easily and much faster than those

from *E. angustifolia* dried roots. Echinacoside yields from SFE residues of both air-dried and freeze-dried roots were 1.13% and 1.47%; cynarine yields were 1.44% and 1.79%, respectively. These results were even higher than those obtained with methanol extraction (Table 2.2, Chapter 2). First, it may be due to the fact that the SFE residues were lighter than the original dried roots because alkylamides and other lipophilic components had been extracted by supercritical CO_2. Secondly, when supercritical CO_2 passed through the roots during SFE, the residue after SFE may have lost some of the moisture and make the residues even lighter than the original dried roots. This resulted in more dry solids in SFE residues being weighed for ethanol extraction compared to the original root powder prior to SFE.

Successful extraction of echinacoside and cynarine from the SFE residues may imply that these two compounds were not subjected to heat damage during SFE at 60°C. These results also proved that supercritical CO_2 could not extract echinacoside and cynarine from *E. angustifolia* roots.

3.4. CONCLUSION

Alkylamides have been successfully extracted using supercritical CO_2. Temperature and pressure of SFE as well as moisture content of the roots had significant effects on the yield of alkylamides. Alkylamides were better extracted at 34 MPa and 60°C from fresh roots, while dried roots resulted in much higher yield at 55 MPa and 60°C. Grinding can significantly increase the yield of alkylamides from dried roots compared with unground dried roots. Ethanol as an entrainer in SFE did not enhance the

alkylamide yields from air-dried roots. There were slightly more alkylamides obtained from freeze-dried roots but they were not significantly different from those of air-dried roots.

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Table 3.1. Particle Size Analysis of Ground Air-dried and Freeze-dried *E.angustifolia* Roots

Mesh No	Particle Size (μm)	Air-dried Roots (weight %)	Freeze-dried Roots (weight %)
16	> 1190	11.61	0.32
20	850-1190	11.45	2.61
40	425-850	25.86	18.25
80	180-425	25.73	44.77
100	150-180	12.84	17.30
Bottom	<150	12.51	16.75

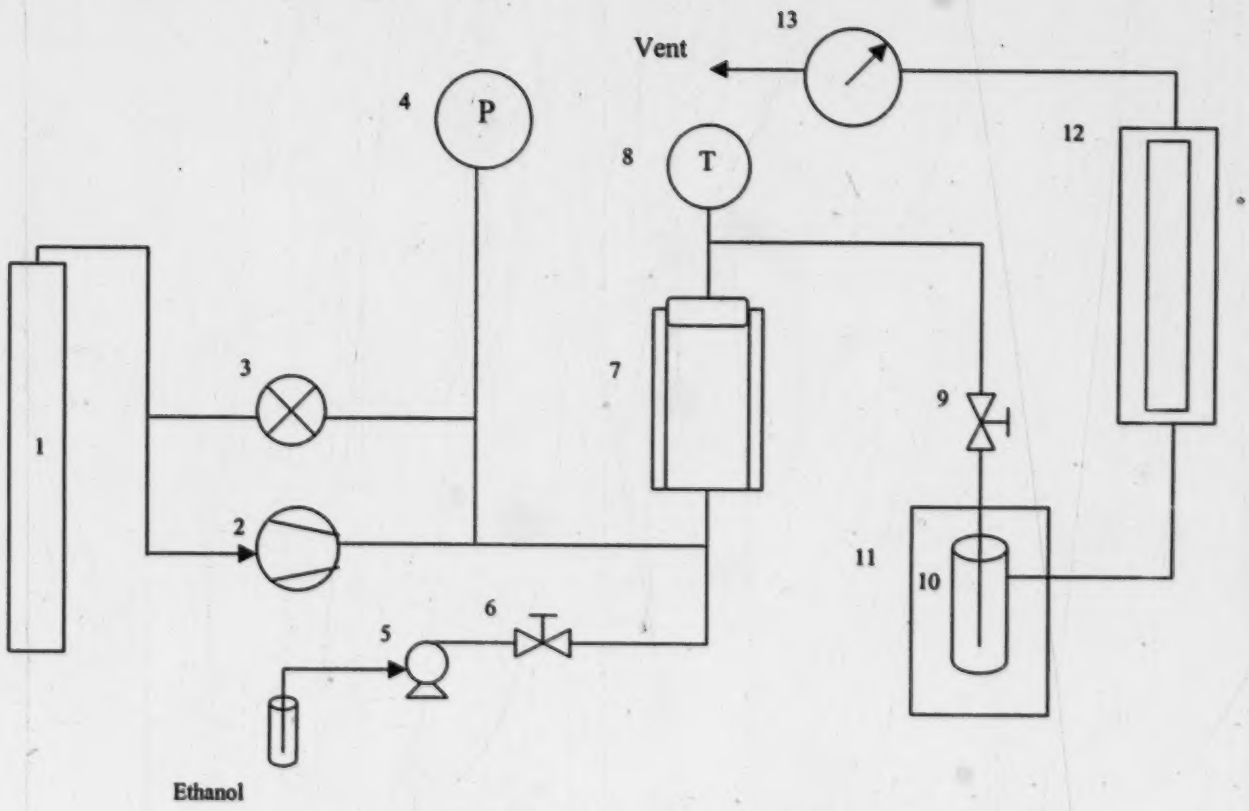


Figure 3.1. Schematic Diagram of the Supercritical Fluid Extraction System

1- CO_2 cylinder, 2- compressor, 3- back-pressure regulator, 4- pressure gauge, 5- Gilson pump, 6- on/off valve, 7- extraction chamber with heater, 8- thermocouple, 9- depressurization valve, 10- extract collection tube, 11- cold bath, 12- rotameter, 13- gas meter.

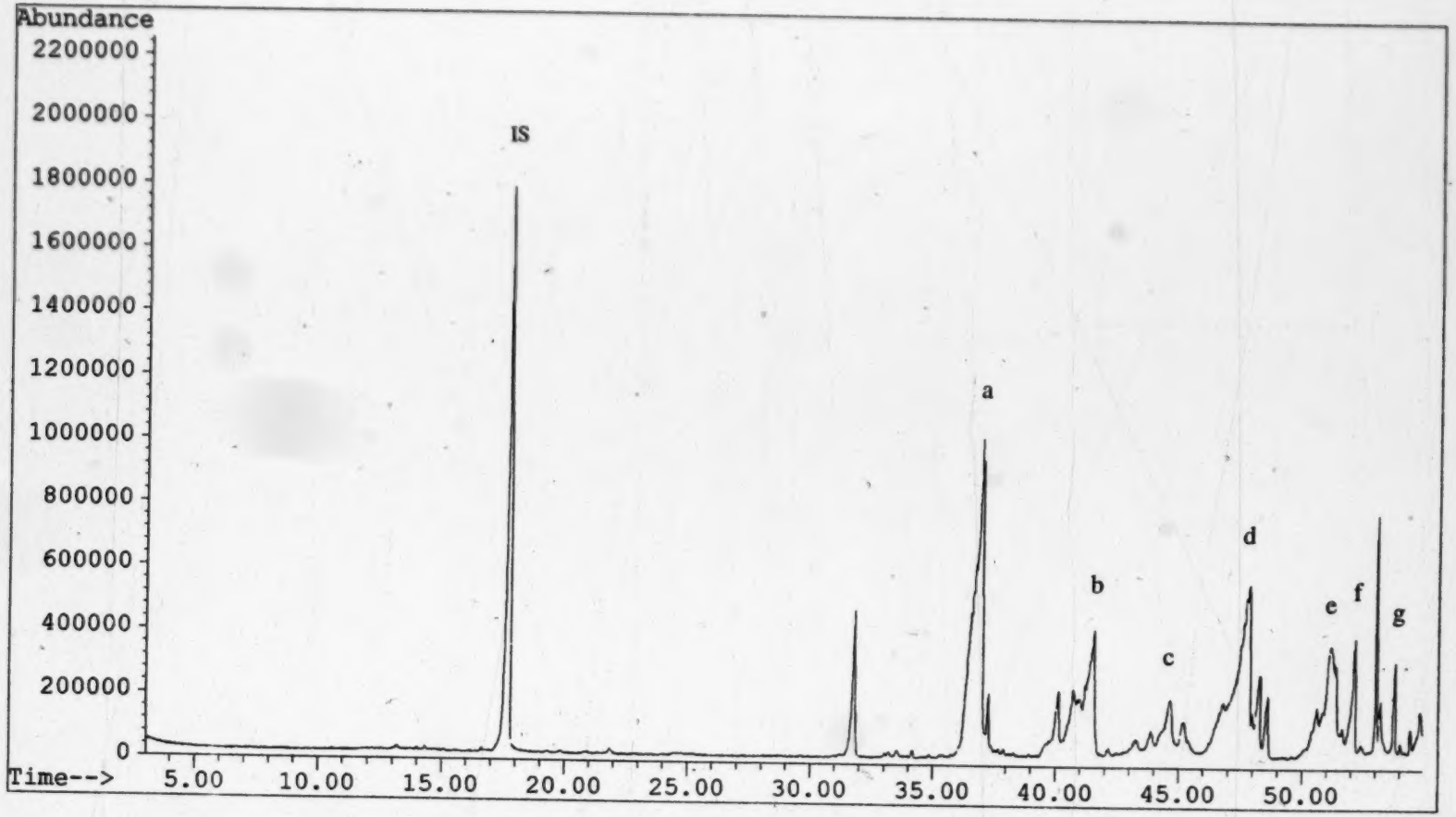
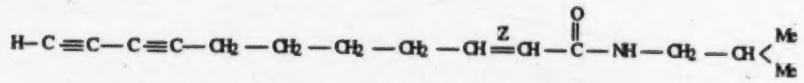
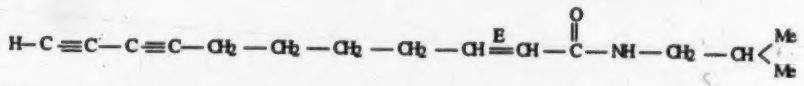


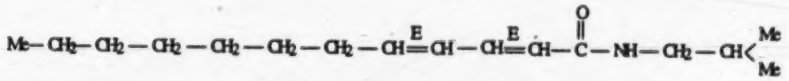
Figure 3.2. Typical Gas Chromatogram of *E. angustifolia* Extracts from Supercritical CO_2 Extraction



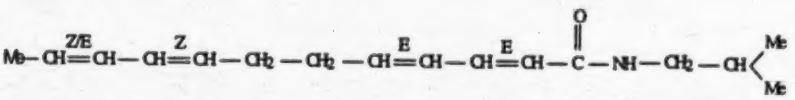
Compound (a), undeca-2Z-en-8,10-diynoic acid isobutyamide, colorless oil



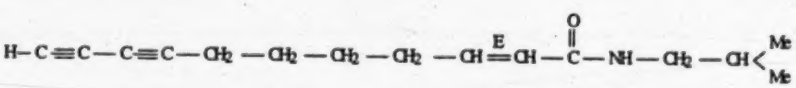
Compound (b), undeca-2E-en-8,10-diynoic acid isobutyamide, colorless crystals



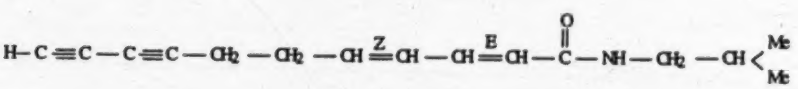
Compound (c), dodeca-2E,4E-dienoic acid isobutylamide, colorless oil



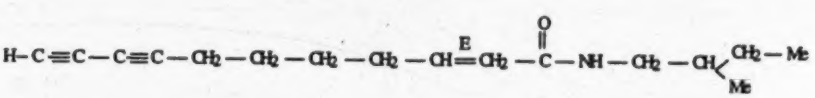
Compound (d), mixture of dodeca-2E,4E,8Z,10Z/E-tetraenoic acid isobutylamide, cystallized as needles



Compound (e), dodeca-2E-en-8,10-diynoic acid isobutylamide, colorless crystals



Compound (f), dodeca-2E,4Z-dien-8,10-diynoic acid isobutylamide, crystals



Compound (g), dodeca-2E-en-8,10-diynoic acid 2-methylbutyamide, colorless oil

Figure 3.3. Chemical Structures of Alkylamides Identified in *E. angustifolia* Extracts

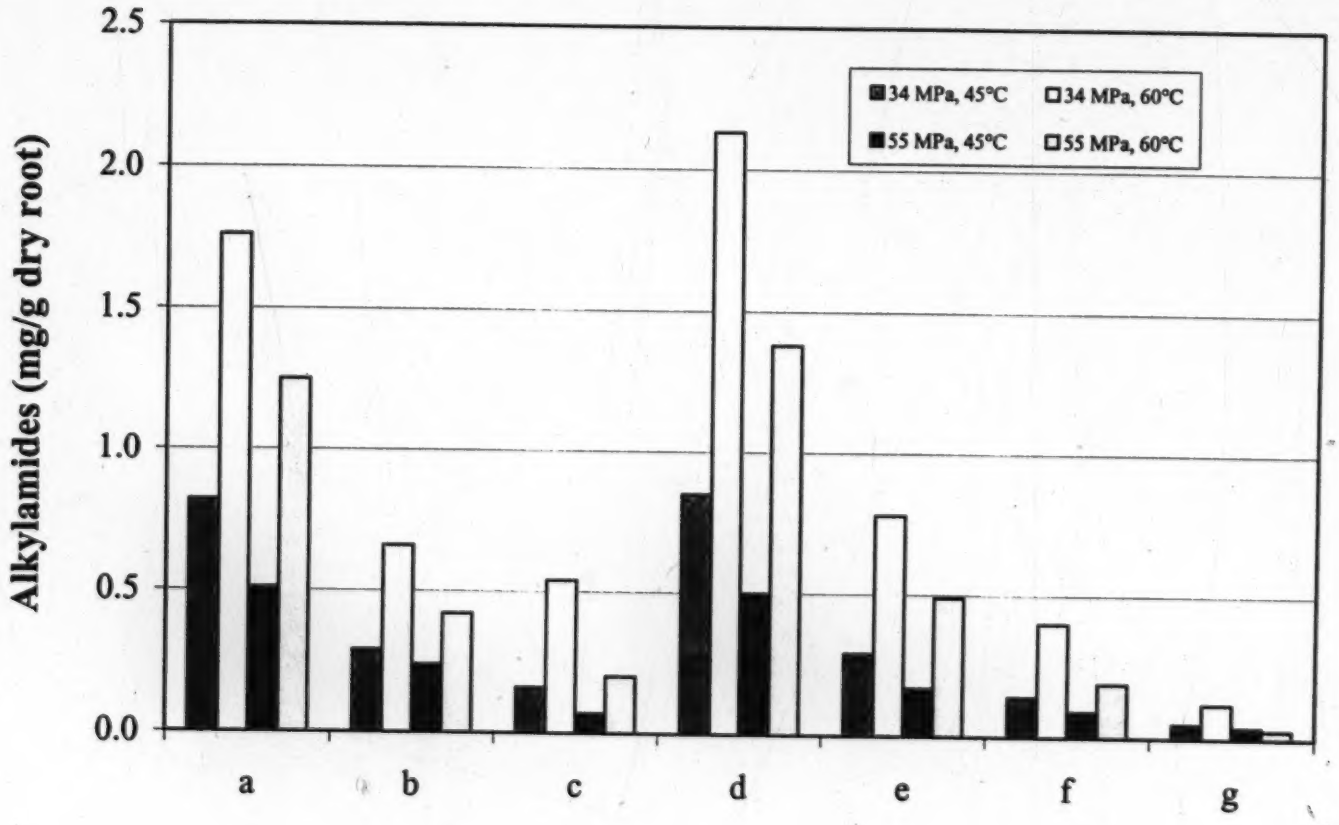


Figure 3.4. Temperature and Pressure Effects on the Normalized Yield of Alkylamides (compounds a-g) in Supercritical CO_2 Extracts of Fresh *E. angustifolia* Roots

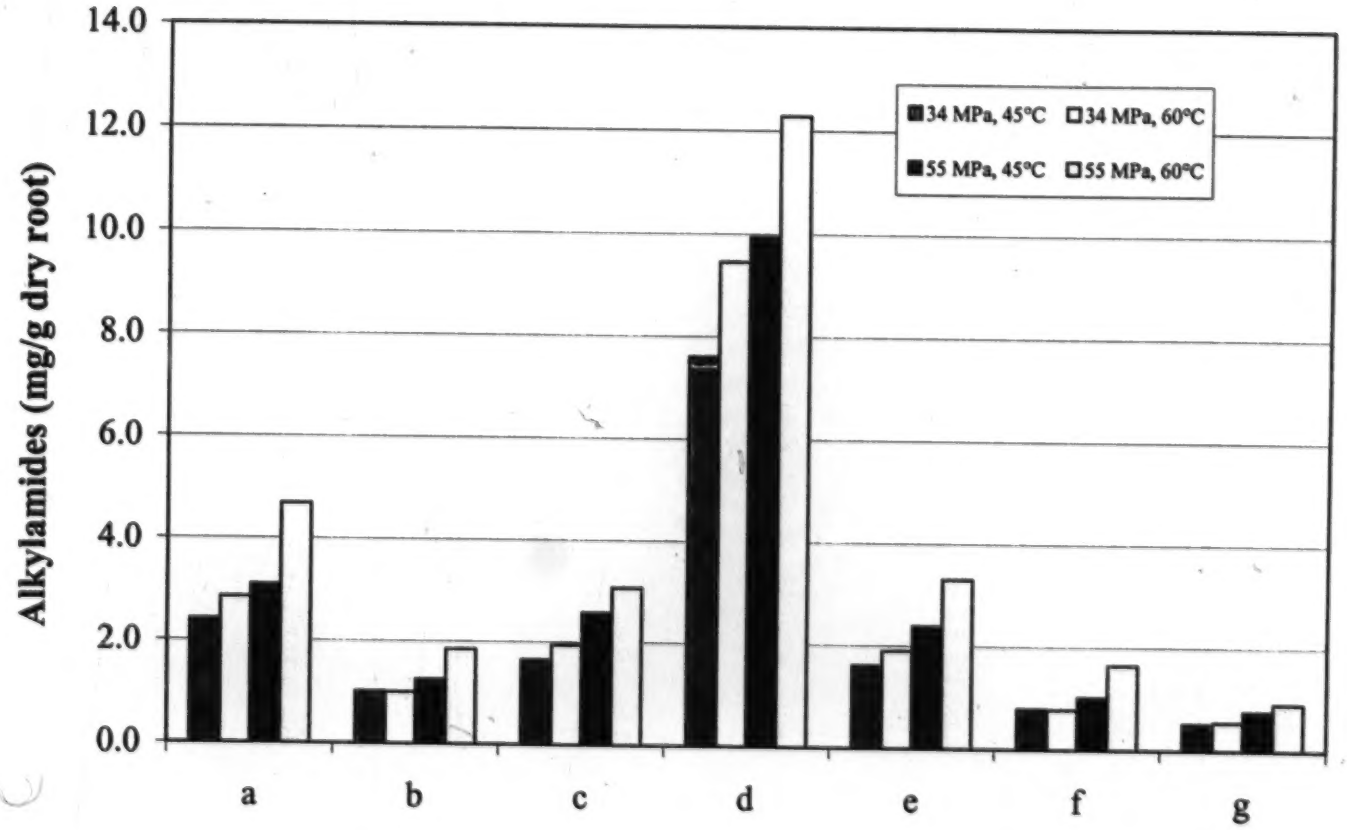


Figure 3.5. Temperature and Pressure Effects on the Normalized Yield of Alkylamides (compounds a-g) in Supercritical CO_2 Extracts of Air-dried *E. angustifolia* Roots

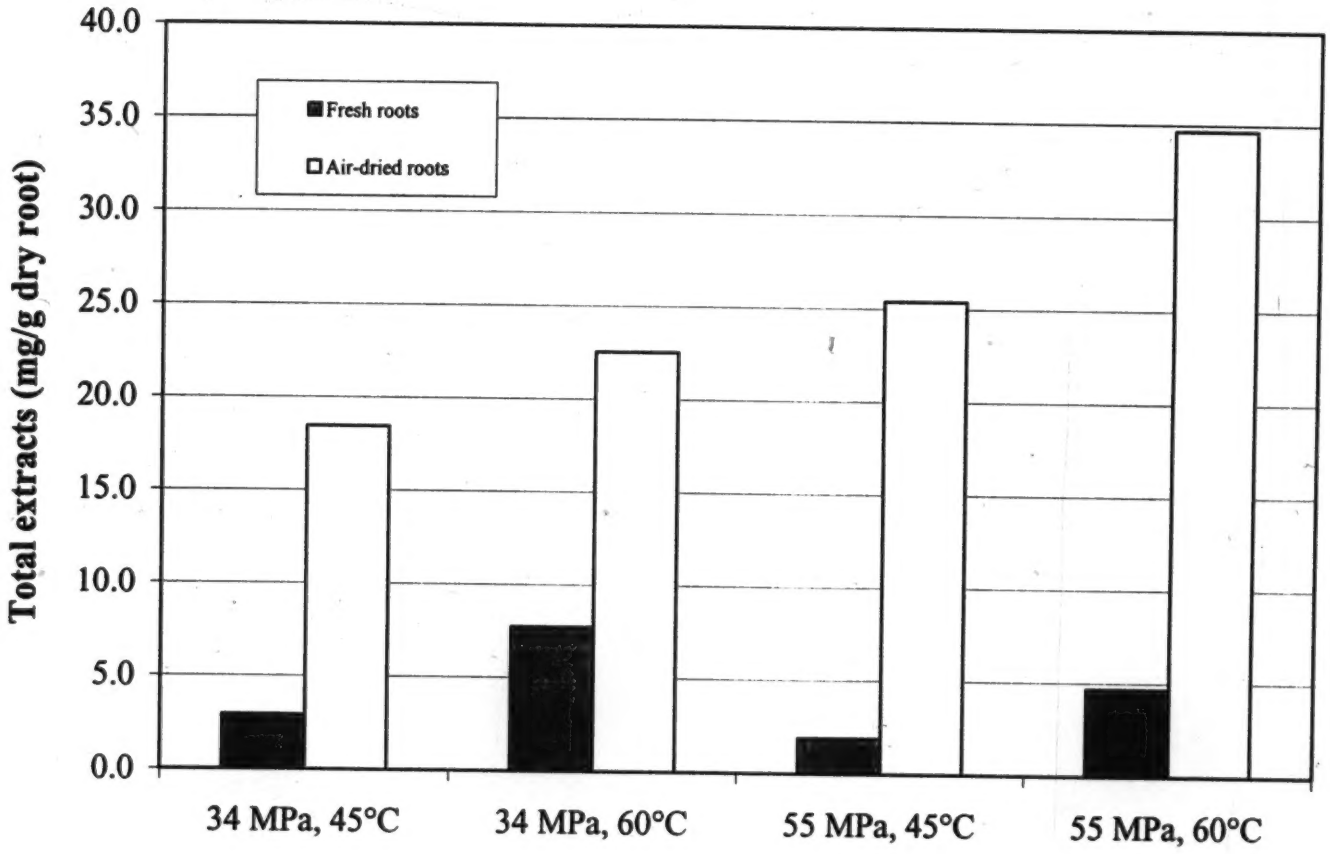


Figure 3.6. Yield of Total Extracts from Fresh and Air-dried *E. angustifolia* Roots Obtained by Supercritical CO_2 Extraction at Different Conditions

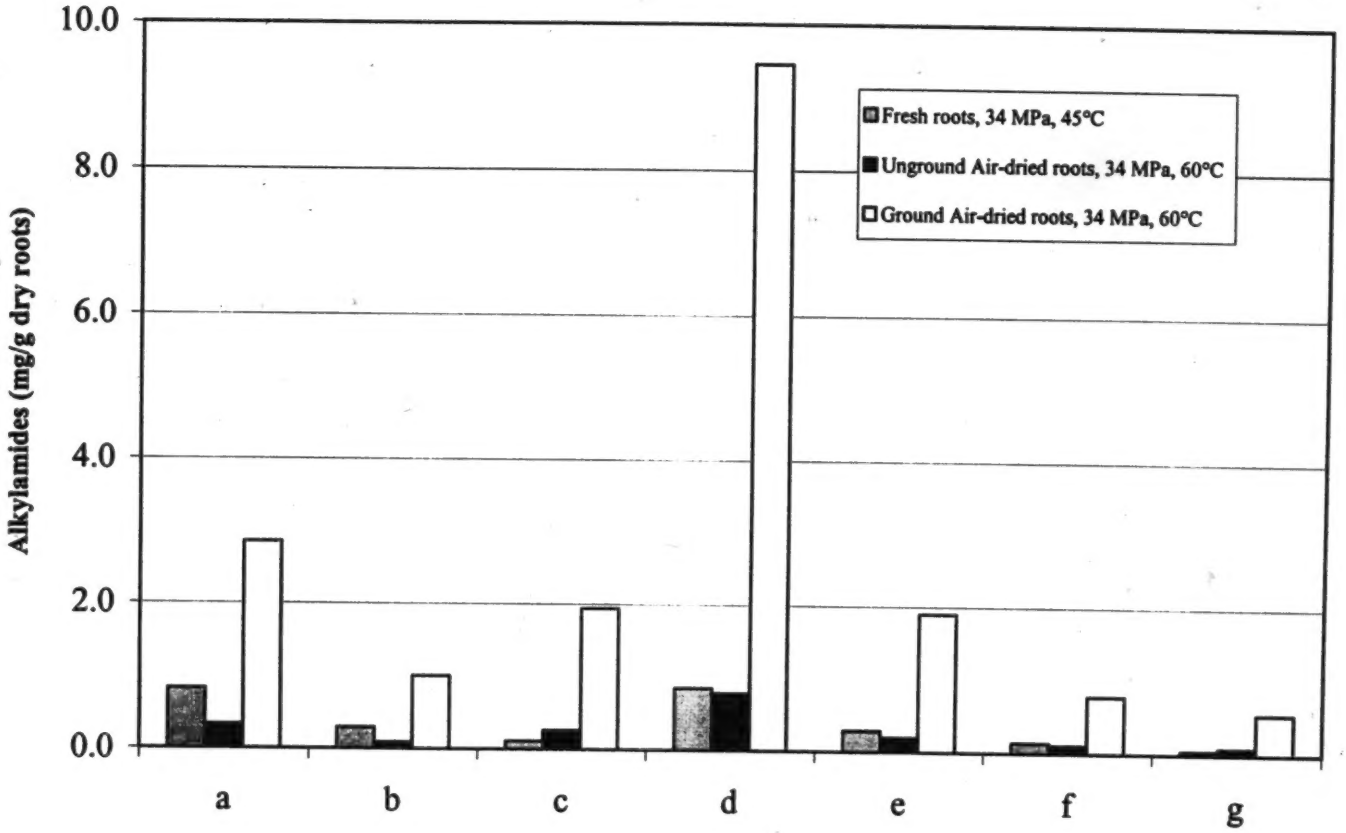


Figure 3.7. Alkylamide (compounds a-g) Yields from Fresh, Ground and Unground *E. angustifolia* Roots Obtained from Supercritical CO_2 Extraction at Different Conditions

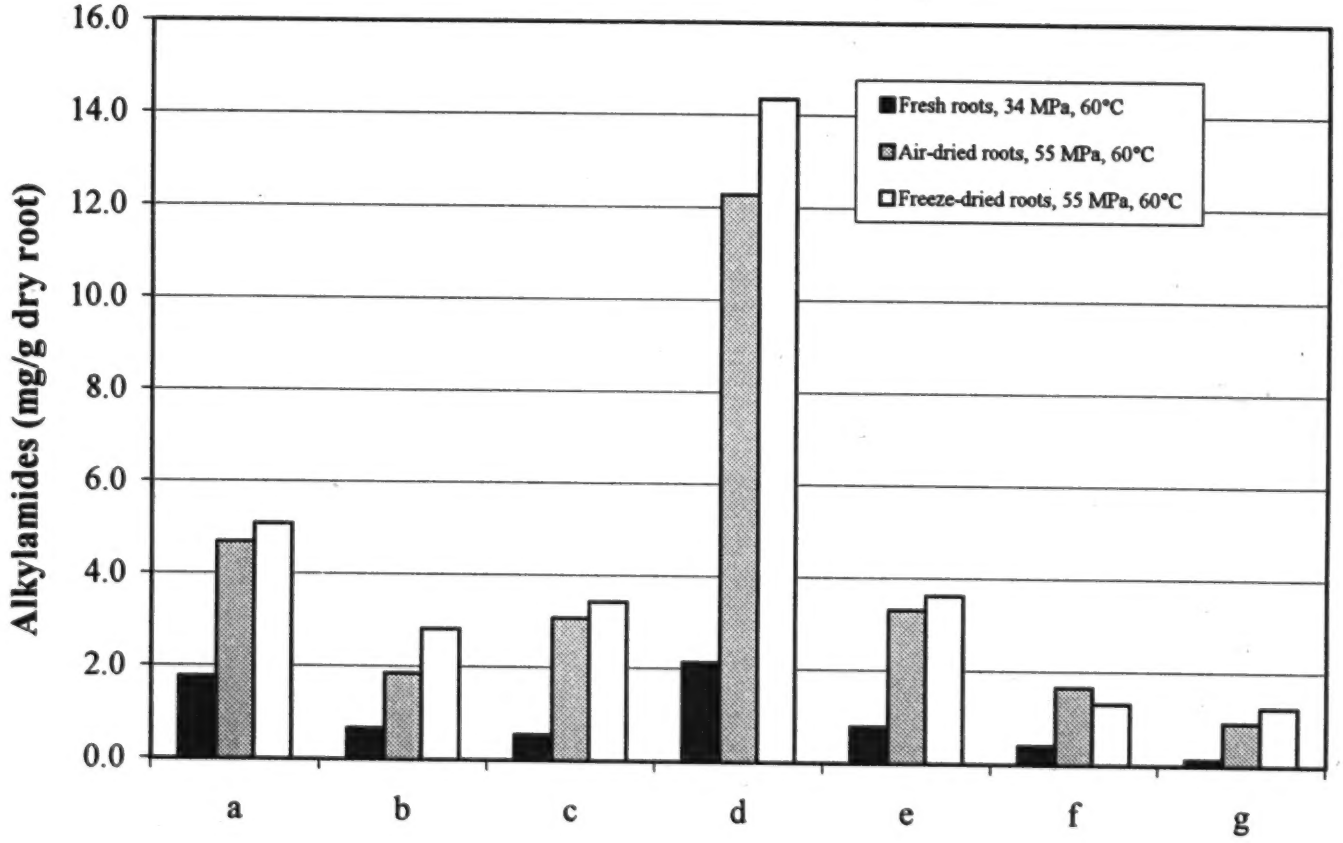


Figure 3.8. Alkylamide (compounds a-g) Yields from Fresh, Air-dried and Freeze-dried *E. angustifolia* Roots Obtained from Supercritical CO_2 at Their Best Extraction Conditions

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. CONCLUSIONS

Caffeic acid derivatives, echinacoside and cynarine in *E. angustifolia* roots were successfully extracted using pure ethanol, while alkylamides were extracted with supercritical CO_2. For echinacoside and cynarine, the best extraction conditions with ethanol were: 75°C (water bath temperature), 90 min extraction time and solvent to feed ratio of 10:1 for echinacoside and 20:1 for cynarine. Longer extraction time appeared to cause degradation of the compounds, resulting in lower yields. Thorough mixing of dry root powder with ethanol was essential for efficient extraction.

For non-polar alkylamides, supercritical CO_2 is a promising solvent for their extraction from the roots. Both pressure and temperature of SFE have significant effects on the individual yield of major alkylamides and total extracts. High temperature and low pressure, i.e. 60°C and 34 MPa resulted in the highest yield of extracts from fresh roots, whereas high temperature and high pressure, i.e. 60°C and 55 MPa gave the best yield of alkylamides from dried roots. Among fresh, ground and unground air-dried roots, ground air-dried sample was the most favorable form for SFE and unground one was the least favorable.

Freeze-dried roots retained slightly higher amounts of echinacoside, cynarine and alkylamides than air-dried roots as indicated by methanol extraction and SFE. However, ethanol extraction of freeze-dried samples gave lower yield of echinacoside and cynarine than those from air-dried roots.

Ethanol extraction of SFE residue produced a much clearer extract, which eluted Sep-Pak more easily and much faster than that extracted directly from dried roots. Therefore, it appears that to maximize the yields of active fractions from Echinacea roots, they should be air-dried, ground and extracted with supercritical CO_2 for alkylamides, followed by ethanol extraction for caffeic acid derivatives, and aqueous extraction for polysaccharides.

4.2. RECOMMENDATIONS

To optimise the yields of echinacoside and cynarine, solvents other than ethanol like acetone should be investigated but special care should be exercised. The extraction and analytical procedures specific to cynarine should also be established for better quantitation. Solvent to feed ratio between 10:1 and 20:1 is suggested for scaling up the ethanol extraction process.

Optimal particle size of ground dried root samples should be investigated with respect to the most efficient ethanol and supercritical CO_2 extractions. Further increase of temperature and pressure within the limits of the supercritical fluid extraction system should be studied further to maximize alkylamide recovery while minimizing degradation of these compounds. A mixture of dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide, which are among the most prominent alkylamides, should be purified and used as standard to facilitate more accurate quantitation of alkylamides. Analysis of alkylamides with GC-MS may be reprogrammed to obtain better peak resolution of alkylamides.